# Snapshots on bacterial tumor colonization

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades einer

Doktorin der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n


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eingereicht am: 14.03.2007
mündliche Prüfung (Disputation) am: 10.07.2007

Druckjahr 2007

# Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

**Publikationen:**

**Westphal K, Loessner H, Leschner S, Weiss S.** Pharmaceutical composition for tumor treatment. EP07102516.7 patent application filed, Feb. 15th, 2007

**Loessner H, Endmann A, Leschner S, Westphal K, Rohde M, Miloud T, Hammerling G, Neuhaus K, Weiss S.** Remote control of tumour-targeted Salmonella enterica serovar Typhimurium by the use of l-arabinose as inducer of bacterial gene expression in vivo. Cellular Microbiology (2007).

**Tagungsbeiträge:**

**Westphal K, Loessner H, Weiss S.** Bacteria as carriers for tumor therapy (Poster), "Molecular Interactions during Infection", Summer School, Quedlinburg (2006).

**Westphal K, Zelmer A, Weiss S.** *Shigella flexneri* mediated gene transfer into established tumors (Poster), "New Approaches to Vaccine Development-From the bench to the field“, Vaccine Congress, Berlin (2005).

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# 1 Introduction

Within the last 100 years the leading causes of death have changed dramatically. Until the middle of the twentieth century death was mainly due to infectious diseases. After the discovery of penicillin by Alexander Fleming in 1928 (reviewed in Bentley, 2005) and the following development of industrially produced antibiotics in addition to improved hygiene, the death toll from infectious diseases has declined drastically (reviewed in Gootz, 1990). Now cancer has become the second-ranking cause of death in the western world after cardiovascular diseases (Fig. 1.1). Thus, the development of a cure for cancer has become a major issue.

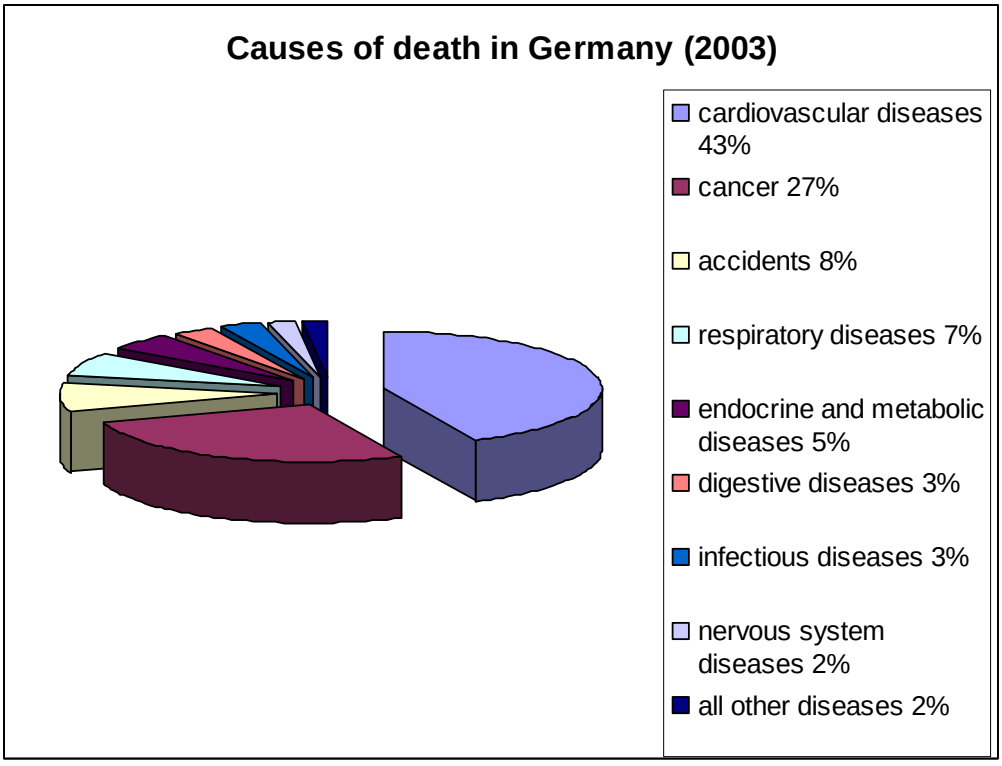



Fig. 1.1: The eight major causes of death in Germany. Source: Federal statistic office Germany. Distribution of death causes is similar in all western countries.

## 1.1 Cancer

What exactly is cancer? “It is easy to forget that cancer is not a single disease, but many diseases” (Bergers and Benjamin, 2003), which is the reason why it is so hard to grasp or understand cancer and to develop a holistic treatment against it. During the past 25 years cancer researchers have enumerated an amazing range of phenotypes and have catalogued thousands of molecular alterations that are associated with cancer and malignant diseases (reviewed in Zhao et al., 2004). Generally, a cancer cell is a cell that undergoes uncontrolled proliferation meaning the cell has escaped normal growth regulating mechanisms. To achieve this, a cell has to undergo several discrete genetic changes, which comprise the alteration of oncogenes, gain of telomerase activity, tissue invasiveness and angiogenesis. A summary of the single stages of tumor progression can be seen in Figure 1.2. (Fuster and Esko, 2005).

Oncogenes are a heterogeneous group of genes that encode proteins capable of inducing cellular transformation. In general, one can define proto-oncogenes or cellular oncogenes (*c-onc*), which are found in normal cells, and viral oncogenes (*v-onc*), which tumor viruses might have acquired from the genome of an infected cell. Most oncogenes are derived from genes that encode growth-controlling proteins.

An example of a retrovirus that carries an oncogene is the Rous sarcoma virus. Its oncogene *v-src* encodes a 60-kDa protein kinase that catalyzes the addition of phosphate to tyrosine residues on proteins. *C-src*, its cellular counterpart, belongs to the multigene family of membrane-associated non-receptor tyrosine kinases and its activity has been shown to be elevated in many human epithelial cancers. C-src is known to increase cellular proliferation. Additionally it seems to promote invasion and motility and thus might contribute to tumor progression. The exact function of c-src in cancer is still unclear. (reviewed in Frame, 2004; Frame, 2002; Yeatman, 2004). Other examples of oncogenes encode growth factor receptors like erbB, which interacts with the major mechanisms of cell death signaling and promotes cell survival (Danielsen and Maihle, 2002). Similarly, *ras* genes encode GTP-binding proteins that are involved in cellular signaling. Mutational activation of Ras proteins also facilitates invasion and metastasis (Giehl, 2005).

Alternatively, oncogenes can act as tumor suppressors, such as p53 (Toledo and Wahl, 2006). P53 is a transcription factor regulating target genes that induce cell-cycle arrest, apoptosis, senescence, DNA repair or alter cell metabolism. P53 is inactivated in 50% of human cancers (Soussi, 2005).

Oncogenes can also regulate programmed cell death like bcl-2. Bcl-2 functions as an oncogene by blocking apoptotic cell death (Piro, 2004; Pattingre and Levine, 2006). However, one mutated or activated oncogene is not enough for a cell to become cancerous. Instead cancer arises from a stepwise accumulation of genetic alterations in multiple oncogenes, resulting in the gradual transformation of a normal cell into malignant derivatives. This multi-step process always coincides with aneuploidy of the tumor cells.

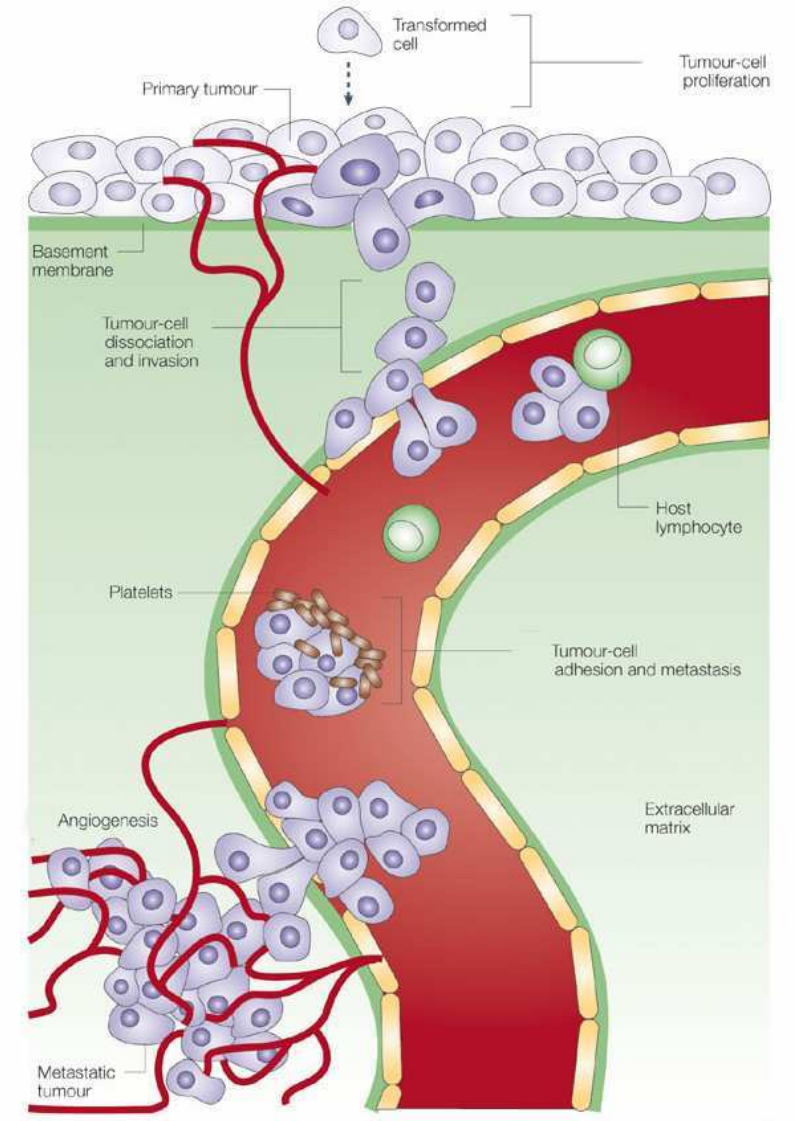



Fig. 1.2: The stages of tumor progression. The single stages are described in the text. The figure is taken from Fuster and Esko, 2005.

Aneuploidy indicates abnormal chromosome content. Normally, a healthy human cell has 23 chromosomes with each chromosome existing as a pair (except sex chromosomes in males). During every cell division, each daughter cell receives the exact set of chromosomes of the parental cell. Therefore, the separation of the two sister chromatids of one chromosome has to be tightly controlled. This control mechanism is called ‘mitotic checkpoint’. A weakening of

the mitotic checkpoint can result in aberrant mitosis, premature separation of sister chromatids during mitosis, merotelic attachment meaning the attachment of one chromatide to both poles and other mitotic checkpoint defects. As a result the cell will end up with irregular chromosome content and become aneuploid (Kops et al., 2005). An example of the spectral karyotype of an aneuploid tumor cell is displayed in Fig. 1.3.

Fig. 1.3: Spectral karyotype (SKY) image of an aneuploid tumor cell. The figure is taken from Pollack, 2006.

Essential for all tumor cells is also the gain of telomerase activity. Telomeres are unique structures at the ends of linear eukaryotic chromosomes. They are composed of repetitive G-rich sequences and are associated with different proteins. Telomeres are responsible for preserving chromosome stability, protection of the chromosome ends against degradation and prevention of chromosomal end fusion (reviewed in Shay et al., 2001).

During every replication step, the telomeres shorten due to the ‘end replication problem’ of linear DNA (Lingner et al., 1995). Thus, for a continuous cell division the telomeres have to be replenished with newly synthesized telomeric repeats, a process, which is accomplished by the reverse transcriptase telomerase. Most animal cells – besides germ cells – normally do not express telomerase to prevent telomere shortening. As a result, the cell will eventually lose telomere length to a point, where the cell cycle arrests and the cell proliferation is stopped. This mechanism is not only a sign of ageing, but it is also an effective tumor suppressor. Concordantly, a hyper-reactive or constitutively expressed telomerase will ultimately support the proliferation of cancerous cells (reviewed in Shay et al., 2001; Bailey and Murnane, 2006).

A cell that has become cancerous will proliferate uncontrolled. As such tumors become large, they require an adequate supply of oxygen and nutrients. In addition an effective way to remove waste products is essential. In a process called angiogenesis, the tumor induces tumor

vascularization i.e. the formation of blood vessels that fulfill their tasks as pipelines for nutrient and oxygen supply and waste removal. Tumor blood vessels architecturally differ from their normal counterparts. They are irregularly shaped, dilated, tortuous and can have dead ends. Often they are leaky and hemorrhagic with a loosely associated endothelial lining (Papetti and Herman, 2002; Bergers and Benjamin, 2003).

Several additional mutations will accumulate during tumorigenesis e.g. via mitotic checkpoint defects and chromosome instability (Kops et al., 2005). This can lead to the capacity of the tumor to degrade basement membranes and extracellular matrix and become invasive. Eventually tumor cells will reach the lymph system and the blood stream. Via this system they will disseminate throughout the body and aggregate with host cells like platelets and lymphocytes and finally lodge in small vessels of distant organs. This will result in the formation of secondary tumors or metastases. Metastases represent the major problem for tumor therapy.

## 1.2 Tumor therapy

Theoretically, most solid tumors can be removed by surgery. However, surgery is not always possible. Tumors might have metastasized to distant organs, where they proliferate although the primary tumor has been removed. In other cases, the tumor might be associated with a pivotal organ and can therefore not be removed. Thus, the activity of an efficient tumor therapy has to be very broad and should reach all tumor cells, including micro-metastases. In addition, an ideal tumor therapy would only affect cancerous cells, without side effects on normal cells or organs.

Routinely used therapies are presently far from fulfilling such criteria. On the other hand, several novel therapies are considered to date. Accordingly, one could distinguish two kinds of tumor therapy: Conventional tumor therapy, which includes surgery, chemotherapy and radiotherapy and innovative tumor therapies, which comprise immunotherapy, therapies with monoclonal antibodies, gene therapies, as well as all kinds of cancer therapies that are presently developed and are not standard in the clinics.

### 1.2.1 Conventional tumor therapy

#### 1.2.1.1 Chemotherapy

The use of chemotherapy as a remedy for cancer began in the 1940s with the use of nitrogen mustard. Based on autopsy findings from soldiers that died of exposure to sulphur mustard gas during the First World War, Goodman and Gilman, pharmacologists at the Yale School of

Medicine, proposed that this compound might destroy lymphoid tumors. Experiments with tumor bearing mice proved them to be right (Gilman, 1963). After a systemic administration of nitrogen mustard they could observe a marked level of tumor regression. Unfortunately, the remission lasted only a few weeks, but the principle that chemicals can be administered systemically to induce tumor regression, was established. Only a few years later, shortly after the Second World War, Syndey Farber at the Harvard Medical School used antifolates to suppress the proliferation of malignant cells in acute lymphoblastic leukemia (Farber, 1948). This was expanded in the following years and decades and a multitude of reagents have been found or developed that effectively manipulate the proliferation of malignant cells to suppress tumor progression. Besides the discovery of natural products like taxanes, combination therapies of different chemotherapeutics have come into focus and have proven to be more effective than single agents (Papac, 2001; Chabner and Roberts, Jr., 2005).

Up to date chemotherapy remains the treatment of choice for most advanced cancers. However, chemotherapy does have several major drawbacks. Most chemotherapeutics work by impairing mitosis, thereby inhibiting cells that undergo fast proliferation. These are, of course, quickly proliferating tumor cells, but also healthy, fast proliferating cells like hematopoietic cells, cells in hair follicles or cells in mucous membranes. Hence, strong side-effects are encountered like hair loss, nausea, diarrhea or anemia. Besides, not all malignant cells are affected by a chemotherapeutic drug. Slowly growing solid tumors can hardly be treated with chemotherapeutics, especially those tumor cells that are located in the center of solid tumors and have stopped division. In addition, some drugs do not have the ability to reach the cells in the center of solid tumors. Other disadvantages of chemotherapeutics are the development of drug resistant tumor cells that stop responding to the chemotherapeutic treatment. This often takes place after repeated treatments with the same drug. In addition, most cytotoxic drugs are carcinogenic themselves. Thus, secondary tumors might be induced by the treatment. (Lake and Robinson, 2005; Chabner and Roberts, Jr., 2005).

Taking all the side effects of chemotherapy together and regarding the poor prognosis especially for the treatment of solid tumors, it becomes clear that chemotherapy alone cannot be the ultimate solution. Other, more specific therapies have to be developed that leave the healthy cells of a body unharmed. One such therapy that has made good progress within the last couple of years, particularly when it comes to solid tumors, is radiotherapy.

#### 1.2.1.2 Radiotherapy

Radiotherapy as a means to treat cancer is even older than chemotherapy. Shortly after Wilhelm Conrad Röntgen discovered X-rays in 1895, they were used on tumor patients in the clinic. Generally, radiotherapy is the use of direct or indirect ionizing radiation to destroy the

DNA of malignant cells and thus control tumor growth. Radiotherapy can be used as primary tumor therapy, but in the majority of cases it is used in combination with surgery or chemotherapy (reviewed in Bucci et al., 2005).

However, it has been a long way from the first trials to treat tumors with radiotherapy to what is in routine nowadays. After numerous trials with X-rays, γ-rays, electrons (microwave technology) and radioactive 'non-permanent' implants, some basic rules were defined:

Firstly, cells that tend to be radiosensitive usually have three properties: they divide rapidly, they do have a long dividing future and they do have an unspecialized phenotype. All three are properties, which are very common among tumor cells (reviewed in Bernier et al., 2004).

Secondly, oxygen is important. There is a direct correlation between radiosensitivity and oxygen. Cells that are well oxygenated tend to be radiosensitive, while cells that are found in hypoxic regions are resistant to radiation. As solid tumors often have a distorted and tortuous tumor vasculature, regions of low oxygen or hypoxia are very common, rendering a large part of the tumor radioresistant. If such cells re-oxygenate after radiation therapy, the tumors can re-grow. This is even more problematic as these are the same cells, that are – due to low oxygen and low nutrient supply – slowly proliferating and thus also resistant to chemotherapy (Gray et al., 1953; reviewed in Bernier et al., 2004).

Finally, a fractionated radiotherapy, in which the given dose of ionizing radiation is divided into fractions and given at different time intervals, is superior to a single high dose of ionizing radiation. As normal, healthy tissue tends to proliferate rather slowly, it has sufficient time to repair the damage that is induced by radiation before replication. In contrast, tumor tissue is rapidly proliferating and does not have enough time to repair the damage. Therefore, the radiation induced DNA-damage can be lethal for tumor cells. In addition, the efficacy of fractionated radiotherapy is increased because of differences in progression of cells through the cell cycle (redistribution), reoxygenation of the tissues and cell division (repopulation) (Withers, 1975; reviewed in Bernier et al., 2004).

Radiotherapy of solid tumors has improved drastically within the last decades, which is mainly due to the rapid development in computer technology that led to three dimensional imaging of the tumors within the body. With computed tomography and magnetic resonance imaging, it is possible to gain a detailed view of how the tumor is located and shaped. Thus, tumors can be treated with exact calculated high doses of radiation with minimized damage of adjacent organs and tissues (Blasberg, 2002; Bucci et al., 2005).

By using hadrons instead of photons, electrons or X- and γ-rays, the outcome of radiotherapy could further be enhanced. Hadrons are subatomic particles that are larger than electrons, such as protons, neutrons and ions. These hadrons are applied as accelerated particle-beams, which

offer an improved dose conformation and a better sparing of normal tissue surrounding the tumor, compared to conventional radiation (Suit et al., 2003; Schulz-Ertner et al., 2006).

However, radiation therapy is still accompanied by side effects. Besides acute, mild side effects like nausea, swelling or diarrhea, the long term side effects can manifest themselves from fibrosis and permanent hair loss to infertility or secondary radiation induced cancers (Bucci et al., 2004; Bentzen, 2006).

These side effects added to the problem that low oxygenated, slowly growing tumor cells are not affected at all clearly point out the need for new or additional cancer therapies. Several preclinical and clinical trials are in progress now that investigate innovative tumor therapies, as they are called in this work, alone or in combination with chemotherapy, radiotherapy and surgery.

### 1.2.2 Innovative tumor therapy

Among the novel tumor therapies that have arisen within the last decades to treat solid tumors a very successful type of therapy stands out. This is the use of humanized monoclonal antibodies (mAbs) against growth factors or their receptors (Hinoda et al., 2004). Trastuzumab (humanized anti-HER2/neu/ErbB-2 mAb) is the first humanized mAb with antineoplastic activity that has passed phase III clinical trials. By its trade name Herceptin, it is approved for the treatment of solid, metastatic breast cancer (Nahta et al., 2004).

Bevacizumab (Avastin), which targets vascular endothelial growth factor (VEGF) and inhibits angiogenesis, has been licensed in the EU in 2005 and is used to treat metastatic colorectal carcinoma (Diaz-Rubio, 2006). Other mAbs on the market are Erlotinib (Tarcerva), which inhibits the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) and is used for cancers of the lung and of the pancreas or Imatinib (Gleevec), which is used for chronic myelogenous leukemia and gastrointestinal stromal tumors. It binds to tyrosine kinase domains in the Abelson proto-oncogene (*abl*), c-kit (CD117) and platelet-derived growth factor receptor (PDGF-R) (reviewed in Collins and Workman, 2006)). Several additional humanized mAbs are still in clinical development with promising perspectives (Reichert and Pavolu, 2004).

By binding to growth factors or their receptors, the therapeutical principle of the mAbs is to shut off the receptor-mediated signaling. Thereby they block cell division and cell growth. However, since all these mAbs target self/tumor antigens they can also influence non-tumor cells and adverse effects like cardiac dysfunction or thrombotic events have been observed (Hinoda et al., 2004).

Another special case of innovative tumor therapy is immunotherapy. In general, immunotherapy is a treatment to stimulate or restore the ability of the immune system to fight infection and disease. A simple method of activating the immune system of a cancer patient is the systemic administration of adjuvants, such as the pro-inflammatory cytokines Interleukin 2 (IL-2) or Interferon-α (IFN-α).

The administration of IL-2 could dramatically increase clinical regression in of melanomas in immunotherapeutic trials (Rosenberg et al., 1998). IL-2 has also been shown to have effects in renal cell carcinoma (RCC) (Margolin, 2000). It is thought that IL-2 facilitates the migration of tumor-antigen (TA)-specific T cells to the tumor site by increasing blood vessels permeability. It further might induce proliferation and activation of effector functions of $CD8^+$ T cells, but also secondary production of pro-inflammatory cytokines (Wang et al., 2004). Similar results have been obtained for IFN-α in melanoma patients (Sabel and Sondak, 2003). Impressive anti-tumor activity was also observed with Bacille Calmette-Guerin (BCG) as a non-specific immune stimulant for non-invasive bladder cancer and lung cancer (see below) (reviewed in Alexandroff et al., 1999; Raez et al., 2005).

Cell based immunotherapies are also amongst the innovative therapies. Trials have been made with so-called lymphokine-activated killer cells (LAK). Peripheral blood cells of patients were pulsed with IL-2 *in vitro* to activate killer cells and re-infused into the patient. While LAK-therapy was effective in some melanoma patients, complete responses were rare. In addition, such cells are difficult to deliver and the priming steps with IL-2 are rather challenging (reviewed in Yannelli and Wroblewski, 2004).

A more directed, specific immunotherapy is adoptive transfer. This method utilizes specific T cells to attack cancer cells via cytotoxic responses. Therefore, T cells that are reactive against a patient's cancer cells are expanded *in vitro* to be adoptively transferred back into the patient. These can be tumor infiltrating lymphocytes from the cancer patient, which do have a natural cytotoxic activity, or genetically engineered T cells (Yee, 2006).

Other approaches aim at the immunization against tumor antigens. Here, tumor lysates or irradiated tumor cells are injected into the patient to induce a specific immune response against the tumor antigens. A variation of this therapy is the use of dendritic cells (DCs) to induce an anti-cancer response. DCs are the best known stimulants of an immune response and therefore an obvious choice for specific initiation of an anti-cancer response. In this case, DCs from cancer patients are differentiated *in vitro* and loaded with tumor-specific antigens. A common method is the administration of purified tumor-antigens or tumor lysate to the DCs. Being loaded with tumor-antigens the DCs are injected into the tumor patients, where they present the tumor antigens and activate effector lymphocytes ($CD4^+$ T cells, $CD8^+$

T cells, B cells and natural killer cells (NK cells)). These cells should migrate to the tumor and exert a cytotoxic activity against the tumor cells (Schott, 2006).

As a special case of immunotherapy, the prophylactic intervention in cancer development should be considered here. The introduction of a vaccine against Hepatitis B has dramatically reduced the incidence of hepatocarcinomas in some developing countries. Probably the continuous inflammation of the liver caused the induction of tumorigenesis (reviewed in O'Brien et al., 2004). Unfortunately, a prophylactic vaccination against cancer is not always possible.

Nevertheless, around 20-30% of all cancers are believed to be induced by pathogens. For instance chronic infections by bacteria are commonplace. Exemplary are *Helicobacter pylori* infections, which are related to the development of gastric cancer (reviewed in Pritchard and Crabtree, 2006). Thus, it might be possible to develop prophylactic therapies or vaccines for several other types of cancer. On the other hand, antigens of such tumor-causing pathogens might represent potential targets for tumor-immunotherapies.

A case that has drawn attention in 2006 was the approval of two vaccines directed against human papilloma virus (HPV), which were designed to immunize against certain sexually transmitted diseases, such as cervical cancer and genital warts that are caused by the virus. Both vaccines have been shown to offer 100 percent protection against the development of cervical cancer. Such an efficiency of a vaccine has rarely been observed so far (Lowy and Schiller, 2006; Lollini et al., 2006). However, the success of most of such trials is rather scarce up to now.

Direct gene therapy is also used to treat cancer. One concept of gene therapy is gene transfer, in which a foreign gene is transferred and inserted into the cancer cells. These can be genes that induce cellular death after expression (suicide genes), genes that inhibit angiogenesis (reviewed in Liu et al., 2006) or genes that lead to the expression of immunostimulatory genes (reviewed in Cross and Burmester, 2006). Genes can be delivered into tumor cells via viral delivery systems like replication incompetent adenoviruses. Alternatively, non-viral delivery systems can be used such as naked DNA that can be transferred into the tumor cells by microinjection, particle bombardment, pressure or electroporation but also via polymeric delivery systems (Polymer-DNA complexes) and liposomal delivery systems, in which the DNA is entrapped in liposomes (reviewed in Patil et al., 2005). These therapies are still highly experimental.

In this context oncolytic virus therapy should be mentioned. Oncolytic viruses have been genetically modified to specifically target to and destroy cancer cells. Usually, these vectors are designed to infect cancer cells and to induce cell death via virus propagation, expression

of cytotoxic proteins and cell lysis. Commonly used are Adenoviruses for prostate cancer. Animal experiments have also been performed with influenza virus or new castle disease virus amongst others. However, these attempts are still in an experimental stage (reviewed in Mullen and Tanabe, 2002; Hemminki et al., 2003).

One very promising non-conventional tumor therapy is bacteria-mediated tumor therapy. It could be combined with gene therapies and immune therapies and could even be used as gene delivery system. Additionally, it specifically aims at the weakly oxygenated, slowly growing tumor cells inside solid tumors, which are not well affected by chemo- and radiotherapy.

#### 1.2.2.1 Bacteria-mediated tumor therapy

The original idea to use bacteria as an anticancer treatment is rather old. Since the middle of the 19$^{th}$ century clinicians have experimented with live bacteria like *Streptococci*, *Mycobacteria* and *Clostridia* to treat human cancer. The first reported treatment with living bacteria as an anti-cancer agent was done in 1868 by the German physician W. Busch (reviewed in Pawelek et al., 2003). He intentionally infected a woman with an inoperable sarcoma by cauterizing the tumor and laying the woman into a bed that was previously used by a patient with "erysipelas", a *Streptococcus pyrogenes* infection. As intended, the woman became infected and rapid tumor shrinkage occurred. Unfortunately, the patient died of the infection nine days later (reviewed in Van Mellaert et al., 2006).

30 years later two surgeons, the American William B. Coley and the German Friedrich Fehleisen, independently began treating cancer patients with *S. pyrogenes* after they both had observed the cure of cancer or at least remarkable tumor regression by severe erysipelas infections. Coley finally dedicated a big part of his life to explore the use of bacteria in anti-cancer therapy. In his later studies he shifted from injecting live bacteria to injecting bacterial extracts that were derived from inactivated *Streptococci* and *Serratia marcescens*, a mixture that is nowadays known as "Coley's toxin", into his patients. He hypothesized that an immune reaction against the infectious material crossreacts with the tumor cells and destroys them, and thus causes the tumor to shrink. Hence, he could be considered the founder of cancer immunotherapy (reviewed in Pawelek et al., 2003; Ryan et al., 2006; Van Mellaert et al., 2006).

However, the beginnings of bacteria-mediated tumor therapy can only be regarded as episodic and hardly be called successful. Many patients became ill or died in succession of the systemic toxicity that was induced by the bacterial infection or the release of necrotic tumor debris. In addition, a complete cure of cancer could rarely be obtained, as a persistent outer

rim of viable tumor cells after bacterial treatment remains (see below). From this, the tumor can re-establish itself (Agrawal et al., 2004).

Some years ago bacteria as a means for specific tumor therapy had a renaissance, when BCG was found to decrease the rates of recurrence of superficial bladder cancer with 70% of the patients responding to BCG (Meyer et al., 2002). BCG is a local bacterial tumor therapy, in which live attenuated BCG are applied directly into the bladder. Up to now BCG is the only bacterial tumor therapy that is successfully used in the clinics. Akaza (1995) showed that 84.4% of 32 cases with CIS (carcinoma in situ) showed a complete response to BCG. BCG is further used for residual papillary tumors to reduce the number and frequency of recurrent tumors and to prevent disease progression. The exact mechanism of BCG induced anti-tumor response is not completely solved yet. Jackson et al. (1994) suggested the induction of an immune response and a tumor response. While the immune response consisted of an inflammatory infiltrate and the secretion of cytokines, they also described CD8 T cells killing via the induction of apoptosis or necrosis. CD4 T cells contribute to the anti-tumor response by the secretion of cytokines leading to the maturation of cytotoxic T cells or possibly more specific BCG activated killer cells (BAK) (reviewed in Meyer et al., 2002) .

Experimentally, bacterial strains of the genera *Bifidobacterium*, *Clostridium*, *Salmonella* and even *Escherichia coli* (*E. coli*) were found to specifically migrate to and preferentially replicate within solid tumors when given systemically (Lemmon et al., 1997; Yu et al., 2004; Sasaki et al., 2006). This intrinsic tumor-targeting feature of the bacteria together with the tremendous improvements in genetic engineering and genomic sequencing of bacterial genomes, have sparked new interests in using bacteria for tumor therapy and opened new possibilities for cancer therapies. Thus, bacteria can be exploited not only as anti-cancer agents, but also as carriers or transporters that could specifically bring chemotherapeutics, toxins, angiogenesis inhibitors and immunotherapeutics like cytokines directly to the place where they are needed: into the solid tumor.

Although many experiments have been performed with different kinds of bacterial strains in order to treat solid tumors, it is still not possible to predict, which bacteria will be successful as therapeutic agents and which bacteria will fail. Bacterial infections always include physiological stress for the host, thus not all bacteria are suitable for tumor therapy.

## 1.3 Suitable bacteria for tumor therapy

Until now, bacteria-mediated tumor therapy has mainly focused on obligate anaerobic species of *Clostridium* and non facultative anaerobic bacteria of the genus *Salmonella*. Due to the

blood vessels in tumors that are structurally and functionally abnormal and result in spatially heterogeneous blood flow (Bergers and Benjamin, 2003), solid tumors are known for their acute and chronic hypoxic regions. As mentioned before, these regions are problematic in chemotherapy and radiotherapy, but they provide optimal growth conditions for anaerobic bacteria. A schematic overview of the microenvironmental regions in a centrally necrotic tumor is visualized in Fig. 1.4.

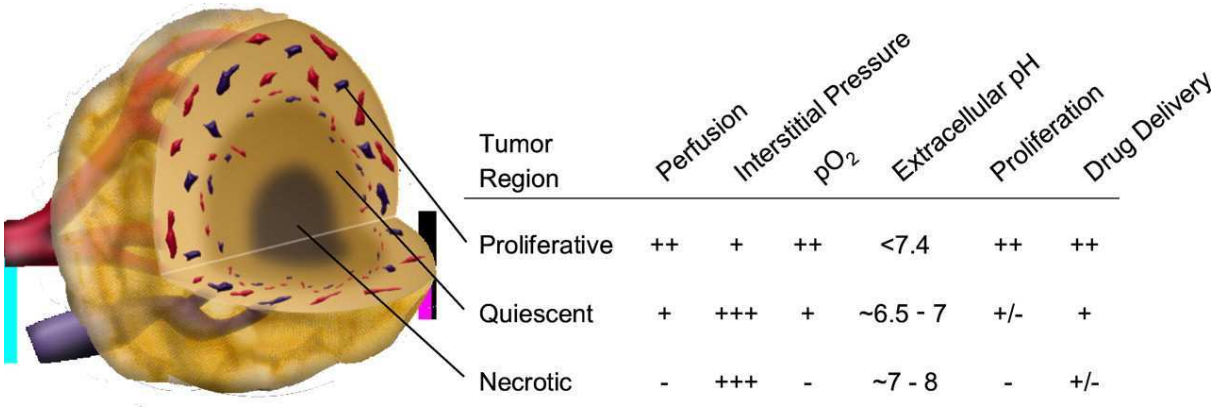



Fig. 1.4: Schematic overview of 3 microenvironmental regions in a centrally necrotic tumor. A spontaneous tumor might consist of more than one necrosis. Decreasing magnitude of various physiological parameters is indicated as +++, ++, +, +/- and -. The figure is taken from Jain and Forbes, 2001.

Certainly, being able to live under anaerobic conditions cannot be the single demand for an anti-cancer bacterium. But what are the criteria that render a bacterium adequate for anti-cancer therapy? In summary, an ideal anti-cancer bacterium should (Jain and Forbes, 2001):

- be non-toxic to the host
- replicate only within the tumor and spare all the healthy tissues and organs of the host
- be motile and disseminate evenly throughout the tumor, including the hypoxic, necrotic and the “healthy” regions of a tumor
- be easily genetically manipulated and easily and stably transformable
- kill tumor cells by competition for nutrients or production of cytotoxins for instance by therapeutic molecules
- slowly and completely be eliminated from the host after treatment
- be of low immunogenicity or non-immunogenic

None of the bacterial strains that are presently in experimental use fulfills all these criteria.

Regarding the requirement for low toxicity for the host, the use of Gram-negative bacteria might represent a problem. The administration of high doses of Gram-negative bacteria can induce tumor necrosis factor alpha (TNF-α) mediated toxic shock. TNF-α is induced upon stimulation with lipid A, a cell wall component of the bacterium. Nevertheless, Gram-negative bacteria can still be favorable for bacteria-mediated tumor therapy. By disrupting the *msbB* gene of *Salmonella*, a gene that is involved in the synthesis of lipid A, Low et al. (1999) reduced the TNF-α induction, thereby generating an increase of $LD_{50}$, the dose at which half of the infected animals die, by 10,000 fold. While enormously reducing the toxicity of this bacterium, the capacity to target into the tumor and suppress tumor growth was retained.

In addition, highly pathogenic bacteria like *Vibrio cholerae* or *Salmonella typhi*, which do have the intrinsic ability to target solid tumors (Yu et al., 2004), represent a high risk for the patient. Thus, they have to be attenuated in a way that they remain invasive but harmless.

More problematic is the search for bacteria that exclusively replicate in solid tumors and spare all the healthy tissues of a patient. Obligate anaerobic bacteria do fulfill this requirement, but they fail in spreading evenly throughout the tumor tissue, as for them well oxygenated areas are not supportive. Thus, facultative bacteria like *E. coli* or some highly attenuated *S. typhimurium* might be more suitable (Forbes et al., 2003; Yu et al., 2004; Saltzman, 2005).

Similarly, requirements of easy genetic modification and transformation are well met by laboratory strains of *E. coli* or *S. typhimurium*. Many obligate anaerobic bacteria are more difficult to be stably manipulated. However, as long as the bacterium has a high delivery capacity for proteins or DNA, it can very likely be genetically modified to satisfy the needs, e.g. the production of cytotoxins (Tjuvajev et al., 2001; Theys et al., 2006). The competition of nutrients is a requirement that is naturally met by every bacterium.

The demand for slow and complete elimination of the bacteria from the host can relatively easily be overcome by using bacteria that quickly respond to antibiotics or by using genetically modified, “conditionally lethal suicide” bacteria that die upon e.g. sugar-induced activation of suicide genes (Davison, 2002; Loessner et al., 2006).

Finally, low immunogenicity of the bacterium used for anti-cancer treatment is required to prevent an immune response against the bacterial carrier itself that is independent of an anti-tumor response. Highly immunogenic bacteria might be eliminated from the host before they reach their destination, especially when the same bacteria have to be used for repeated treatments.

### 1.3.1 Obligate anaerobic bacteria

Among the obligate anaerobic bacteria that have been used for tumor-targeting are mainly species of the genii *Clostridium* and *Bifidobacterium*.

*Clostridia* are often found in tumors of patients suffering from colon carcinomas. In fact, presence of *Clostridia* in a patient can be diagnostic for such neoplasia (Myers et al., 1992). Experimentally, the first attempts have been performed with spores of the Gram-positive bacterium *Clostridium tetani* (*C. tetani*) that were injected into normal and tumor-bearing mice. All tumor mice died of tetanus poisoning due to toxin production by growing vegetative cells, while the healthy control mice remained unaffected. On one hand this had proven that the spores required anaerobic conditions to germinate. Such conditions are present only in solid tumors. On the other hand it demonstrated the strong selectivity of the spores for hypoxic areas of the tumors and its general applicability for tumor therapies (Malmgren and Flanigan, 1955). Recent studies explored spores of non-pathogenic *Clostridia* such as *C. novyi*-NT, a *C. novyi* strain devoid of lethal toxin or *C. sordellii* (Dang et al., 2001; Agrawal et al., 2004).

As mentioned already, the spores need low oxygen partial pressure, since they germinate only within the hypoxic regions of solid tumors. Thus, colonization by *Clostridia* is limited to large solid tumors, in which blood supply is not sufficient to provide the complete tumor with oxygen (Barbe et al., 2006).

The colonization of the tumor with *Clostridia* always led to an increase in the size of necrosis. This limits tumor growth. However, due to a remaining rim of viable tumor cells, the tumor tends to restart growth from such viable tumor cells. To improve bacterial tumor therapy with *Clostridia*, *C. novyi*-NT has been used in combination with chemotherapeutics (Dang et al., 2001) and with radiotherapy (Bettegowda et al., 2003). In both cases the supplementary treatment with the bacteria leads to an increased anti-tumor activity of the treatment.

Other approaches utilized living *Clostridia* that were engineered to produce anti-cancer agents such as TNF-α. This cytokine is highly toxic when given systemically. Being encoded under a bacterial promoter that is induced by radiation, tumor-specific secretion of TNF-α could be demonstrated (Theys et al., 1999).

*C. histolyticum* and *C. sporogenes* were both engineered to carry *E. coli* cytosine deaminase (CD). CD is a so-called prodrug converting enzyme that is able to convert the non-toxic prodrug 5-fluorocytosine (5-FC) into the cytotoxic drug 5-fluorouracil (5-FU) (Theys et al., 2001; Liu et al., 2002). The mechanism of the conversion of 5-FC into 5-FU is depicted in Fig. 1.4. Likewise, *C. acetobutylicum* was engineered to secrete biologically active rat IL-2 as an anti-cancer agent. However, this strain has not been tested *in vivo* yet (Barbe et al., 2005).

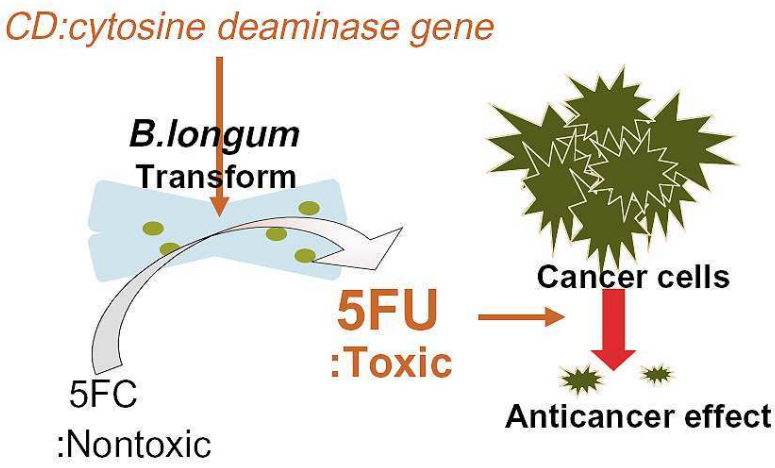



Fig. 1.5: Schematic representation of bacteria-mediated cytosine deaminase therapy with *B. longum* as a model bacterium. The figure is taken from Fujimori, 2006.

Similar treatments were carried out with the non-pathogenic, Gram-positive domestic bacterium *Bifidobacterium longum* (*B. longum*). *B. longum* was shown to colonize large solid tumors upon systemic infection, similar to *Clostridia*. Genetically engineered *B. longum* were successfully used in chemically induced mammary rat tumors (Yazawa et al., 2001). One such approach also exploited the enzyme-prodrug therapy with *E. coli* CD as has been described for *Clostridia* (Sasaki et al., 2006), (compare Fig. 1.5).

*Bifidobacterium adolescence* was transformed with a plasmid encoding the endostatin gene. Endostatin is a broad spectrum angiogenesis inhibitor that might interfere with angiogenesis promoting growth factors such as VEGF (reviewed in Folkman, 2006). This bacterium could target subcutaneously implanted Heps liver tumors in BALB/c mice, and inhibited angiogenesis and tumor growth (Li et al., 2003a).

A major disadvantage of such obligate anaerobic bacteria is that they are only able to target large solid tumors and leave small tumors and metastases unaffected (Pawelek et al., 2003; Ryan et al., 2006). This is due to the fact that small tumors are usually well vascularized and hence have no areas of hypoxia and necrosis. Additional measures have to be employed to reach the inhibition of angiogenesis and the induction of necrotic areas in smaller solid tumors. Therefore, obligate anaerobic bacteria will most likely never be able to distribute evenly throughout a solid tumors. If not exclusively designed as a supplementary treatment for chemo- or radiotherapy, tumor-targeting bacteria should be able to tolerate various conditions. Interestingly, even facultative anaerobic, tumor-targeting species like *S. typhimurium* or *E. coli* do not settle outside the tumor necrosis although they should not be affected by oxygen supply.

### 1.3.2 Facultative anaerobic bacteria

When looking for an ideal anti-cancer bacterium that is at least theoretically able to meet all the mentioned demands for a perfect anti-cancer bacterium, facultative anaerobic bacteria seem to be the preferable choice for such therapies. Provided that a strain can be established that disseminates throughout solid tumors and fulfills most of the other mentioned criteria. Possible facultative anaerobic bacteria as candidates for anti-cancer therapies include *E. coli*, *Salmonella* species and *Shigella flexneri* (*S. flexneri*).

#### 1.3.2.1 *Escherichia coli*

A prime candidate for bacteria-mediated tumor therapy is *Escherichia coli*. *E. coli* is a Gram-negative, rod-shaped, facultative anaerobic bacterium, which belongs to the group of enterobacteriaceae (Schlegel, 1992). It was discovered in 1919 by the German Theodor Escherich as one of the main species of bacteria that lives in the lower intestines of mammals. Today it belongs to the best investigated organisms in the world. Originally, *E. coli* is a commensal bacterium that colonizes the gastrointestinal tract of human infants within the first hours after birth, where it coexists with its human host in mutual benefit for decades. Inside the gastrointestinal tract it resides in the mucous membrane of the mammalian host (Nataro and Kaper, 1998; Kaper et al., 2004). Very rarely commensal *E. coli* are able to cause disease in immunocompromised hosts or when the gastrointestinal barriers break down. On the other hand, several highly adapted *E. coli* strains have evolved that cause a broad spectrum of human diseases, characterized by three different clinical syndromes: urinary tract infections, sepsis/meningitis and enteric/diarrheal disease.

Uropathogenic *E. coli* (UPEC) infect the host via the urethra. They are associated with cystitis and pyelonephritis in the normal urinary tract, which include the expression of P fimbriae, hemolysin, aerobactin, serum resistance and encapsulation (Manges et al., 2001).

Meningitis/sepsis-associated *E. coli* (MNEC) is a pathotype that is the most common cause for Gram-negative neonatal meningitis. This strain is spread hematogeneously (Dawson et al., 1999; Kaper et al., 2004).

The intestinal pathogens are further divided into six categories: enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), entero-aggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Each of the diarrheagenic *E. coli* has unique features in their interaction with eukaryotic cells (Nataro and Kaper, 1998; Torres et al., 2005). The unique feature of each of these bacteria is shown in Figure 1.6.

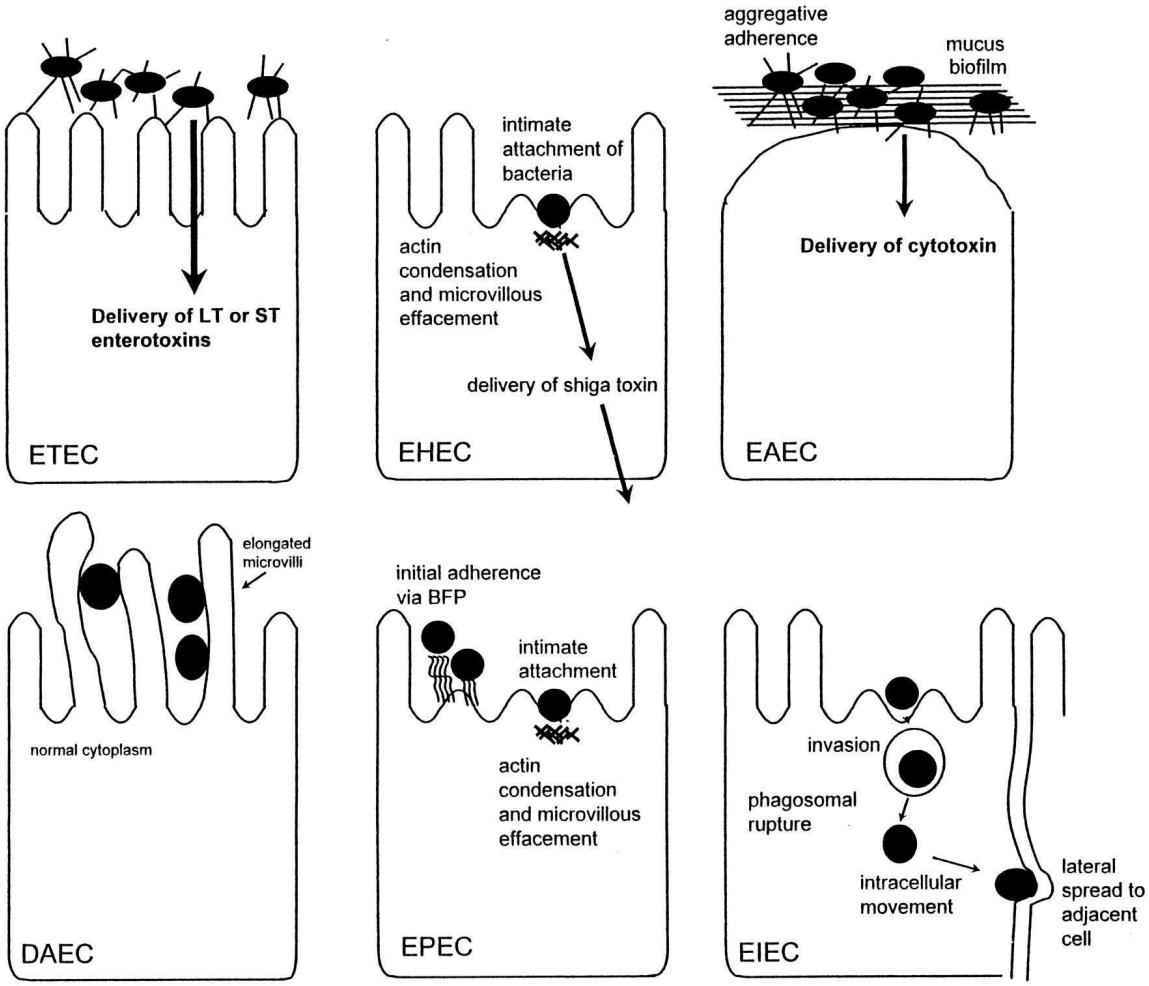



Fig. 1.6: Pathogenic schemes of diarrheagenic *E. coli.* The six different categories of enterophatogenic *E. coli* use different mechanisms to interact with the host cells. These interactions are described in more detail in the text. The descriptions are based on *in vitro* studies and do not necessarily completely reflect the *in vivo* mechanism. The figure is taken from Nataro and Kaper, 1998.

ETEC strains adhere to small bowel enterocytes at the surface of the small bowel mucosa and induce watery diarrhea via the secretion of heat-labile (LT) and/or heat-stabile (ST) enterotoxins (Spangler, 1992; Wolf, 1997).

EHEC adhere to enterocytes in the colon and destroy the normal microvillar architecture by inducing an attaching and effacing lesion. EHEC are further known for the elaboration of Shiga toxin as their major virulence factor, which, after systemic absorption, leads to potentially life-threatening complications (Reid et al., 2000; Perna et al., 2001).

EAEC adhere to small and large bowel epithelia as a thick layer of auto-aggregating bacteria that deliver secretory enterotoxins and cytotoxins (Hicks et al., 1996; Nataro and Kaper, 1998).

DAEC elicit a characteristic signal transduction effect in small bowel enterocytes that manifests as growth of long, finger-like projections, which wrap around the bacteria (Bilge et al., 1989; Bernet-Camard et al., 1996).

Similar to EHEC, EPEC induce the attaching and effacing of lesions in enterocytes, but in the small bowel. They destroy the normal microvillar architecture and induce the characteristic attaching and effacing lesion (McDaniel et al., 1995; Kenny et al., 1997).

EIEC actively invade the colonic epithelial cell, lyse the vacuole and move through the cell by nucleating actin microfilaments. These bacteria can move laterally through the epithelium by direct cell-to-cell spread or by exiting the cell and entering the adjacent cell via the baso-lateral plasma membrane (Pupo et al., 2000; Sansonetti, 2002; Wei et al., 2003).

Besides the commensal type in the gut of mammals and the numerous potentially pathogenic species, *E. coli* strains exist, which have beneficial therapeutic effects for their host. One such strain is the probiotic *E. coli* Nissle 1917, which was isolated from the stool of a soldier by Dr. Alfred Nissle. This bacterium had prevented the soldier from enteritis, while all the comrades of the soldier suffered from the disease (Nissle, 1918). In the meantime, *E. coli* Nissle 1917 is applied as Muafluor® for probiotic treatment for diarrhea. It also showed success in the treatment of diseases like ulcerative colitis, Crohn's disease and inflammatory bowel disease (Kruis et al., 1997; Malchow, 1997; Kruis, 2004). Recently, *E. coli* Nissle 1917 was explored for targeted delivery of molecules to the intestine. When genetically engineered to display a model peptide on the bacterial surface, this strain proved to be useful for localized delivery of molecules into the intestine in cases where an immune response against the carrier strain and its product is undesired (Westendorf et al., 2005).

Independent of the pathological and probiotic potential of some strains, *E. coli* has become *the* laboratory microorganism. Having been isolated from the stool of a convalescent diphtheria patient in 1922, *E. coli* K12 was repeatedly passaged for 50 years in the pathology department at Stanford University before various strains were typed and categorized (Bachmann, 1972; Fux et al., 2005). The genome of an *E. coli* K12 laboratory strain, which had been relieved of the temperate phage lambda by UV irradiation and of the F plasmid by acridine orange, has been fully sequenced (Blattner et al., 1997). A plethora of defined mutations have rendered this enterobacterium a non-pathogenic tool in the laboratory, which is easy to handle and to genetically modify. Besides the described manipulations of *E. coli* Nissle 1917, laboratory strains of *E. coli* were shown to be usable in many clinical applications, including anti-cancer therapies.

In 2004 Yu et al. could demonstrate that the laboratory strain *E. coli* DH5α was able to home to and proliferate within solid tumors of nude mice after a systemic administration. This was

extended to tumor-bearing immunocompetent Lewis rats. Similar to systemic infections with attenuated *S. typhimurium*, *Vibrio cholerae* and *Listeria monocytogenes*, light-emitting *E. coli* were shown to naturally home to primary tumors, but also to small metastatic nodules. The authors proposed that all tested bacterial strains enter the tumors through leaky vasculature and remain there to escape the host's immunosurveillance and find refuge in the tumor tissue.

Half a year later, Critchley et al. (2004) were able to prove *in vivo* protein delivery of β-galactosidase, a hydrolase enzyme that catalyzes the hydrolysis of beta-galactosides into monosaccharides, by recombinant, invasive *E. coli* into the tumor cells of a subcutaneous B16 tumor. These *E. coli* were expressing invasin from *Yersina pseudotuberculosis* to selectively invade non-phagocytic cells, in which $\beta_1$-integrin is expressed and accessible. Although Critchley et al. injected the recombinant *E. coli* directly into the tumors of the mice their experiments highlighted the possible therapeutic applications for *E. coli* as drug delivery vectors into solid tumors.

Other *E. coli* based anti-cancer strategies explored recombinant, invasive *E. coli* as oral vaccines, which are taken up from the intestinal lumen to the Peyer's Patches, where they co-localize with DCs and B cells. These bacteria were modified to co-express model antigens, which resulted in systemic protection against a lethal challenge with antigen expressing tumor cells (Critchley-Thorne et al., 2006).

#### 1.3.2.2 *Salmonella enterica* serovar Typhimurium

A presently often employed candidate for bacteria-mediated anti-cancer therapy is *Salmonella enterica* serovar Typhimurium. *S. typhimurium* is a facultative anaerobic, Gram-negative, rod-formed bacterium that belongs to the group of enterobacteriaceae (Schlegel, 1992). The genus *Salmonella enterica* comprises diverse groups of *Salmonella*, which are the causative agent of infections of the lower intestine in animals and man. The severity of the infections varies depending on the infecting strain and can result in life-threatening typhus. *S. enterica* serovar Typhimurium serves as experimental mouse model for human typhoid fever. In humans typhus is caused by *S. typhi* (Pang et al., 1998), while *S. typhimurium* only causes enteritis.

Being ingested with contaminated food, *Salmonellae* enter the body by invading the intestinal mucosa. Therefore, they have to cross the mucosal surfaces of the gastrointestinal tract. In the natural course of infection, *Salmonellae* target selectively, but not exclusively, M cells, a unique epithelial cell type that is specialized for transepithelial transport of macromolecules. M cells are located in the follicle associated epithelium (FAE) that covers the small intestine and the colon (Clark et al., 1994; Neutra et al., 1996). Here, the *Salmonellae* cause active ruffling of the apical surface of the M cell, which eventually leads to a disassembly of the

apical cytoskeleton and the engulfment of the bacteria into membrane bound vesicles, a process that is called macropinocytosis (Finlay et al., 1991; Bliska et al., 1993; Jones et al., 1993; Clark et al., 1994; Jones et al., 1994). In contrast to other enteropathogenic bacteria, *Salmonellae* do survive and replicate within the phagosome, but they are unable to escape this compartment. After rapid transcytosis, *Salmonellae* are transported into the transepithelial pocket, where they are taken up by professional phagocytes like macrophages, neutrophils and DCs. Within these cells, genes of SPI1, the *Salmonella* pathogenicity island 1, are activated. These genes are representing the invasion system of *Salmonella*. SPI1 encodes a type III secretion system that is able to inject bacterial proteins into the cytosol of the infected phagocytic cell (see below). Thereupon caspase-1, a cysteine-aspartic-acid-protease, is activated. This results in killing of the infected macrophages in a process similar to apoptosis. As a consequence the engulfed bacteria are released and inflammation is initiated. By activation of SPI2, another pathogenicity island, which encodes an alternative type III secreton, *Salmonellae* evolved a strategy to survive inside macrophages. This might facilitate dissemination of the bacteria in the host. As can be seen in Figure 1.7, *Salmonellae* are also able to infect epithelial cells directly (2) and they can be captured by DCs at the mucosal surface and are then transported from the gastrointestinal tract to the bloodstream by macrophages (3) (reviewed in Mastroeni and Menager, 2003; Abrahams and Hensel, 2006).

The gastroenteritis that is associated with *Salmonella* infections is caused by the irritation of the mucous membrane in response to the release of endotoxin, a lipopolysaccharide of the bacterial cell wall.

Beside its pathogenic characteristics during its natural route of infection, *Salmonella* species have been shown to exhibit a promising potential as therapeutic agent for cancer- and immunotherapies. As *Salmonella* species are closely related to *E. coli*, they can be similarly genetically modified like their laboratory relative. Vectors that are designed in *E. coli* laboratory strains can be directly transformed into and used by *S. typhimurium* in the mouse model. In addition, the full sequence of *S. typhimurium*'s genome is known (Sanderson et al., 1995), which facilitates genetic manipulations. Therefore, *Salmonellae* have been extensively used as a delivery system for heterologous antigens and for the construction of multivalent vaccines (Lee et al., 2004; Bauer et al., 2005; Avogadri et al., 2005; Xiang et al., 2005; Lee et al., 2005). They can also be manipulated to deliver proteins to host tissues and malignant tumors. King et al. (2002) for instance developed an attenuated *S. typhimurium* strain that expresses *E. coli* CD. This strain accumulates in solid tumors when given systemically. After a systemic administration of 5-FC to mice bearing an adenocarcinoma, tumor-specific conversion into the cytotoxic drug 5-FU was observed and resulted in inhibition of tumor growth. Other researchers (Saltzman et al., 1996; Saltzman et al., 1997) manipulated

attenuated *S. typhimurium* to express human interleukin 2 (IL-2), a 15 kDa cytokine that was described to stimulate the host's immune system (compare chapter **1.2.2**). Naturally it is produced by activated $CD4^+$ T cells, promotes lymphocyte killing and enhances the cytolytic activity of T cells and NK cells. Systemically administered, high doses of IL-2 caused severe toxicities in a number of patients, which include malaise, fever, anasarca, jaundice, renal dysfunction and capillary leak syndrome (Kintzel and Calis, 1991; Saltzman, 2005). In contrast, local production of IL-2 by attenuated *S. typhimurium* inside solid tumors did – at least in animal models – cause only diminished toxicities. At the same time significantly fewer neuroblastoma metastases were found and significantly smaller neuroblastomas (Saltzman et al., 1996).

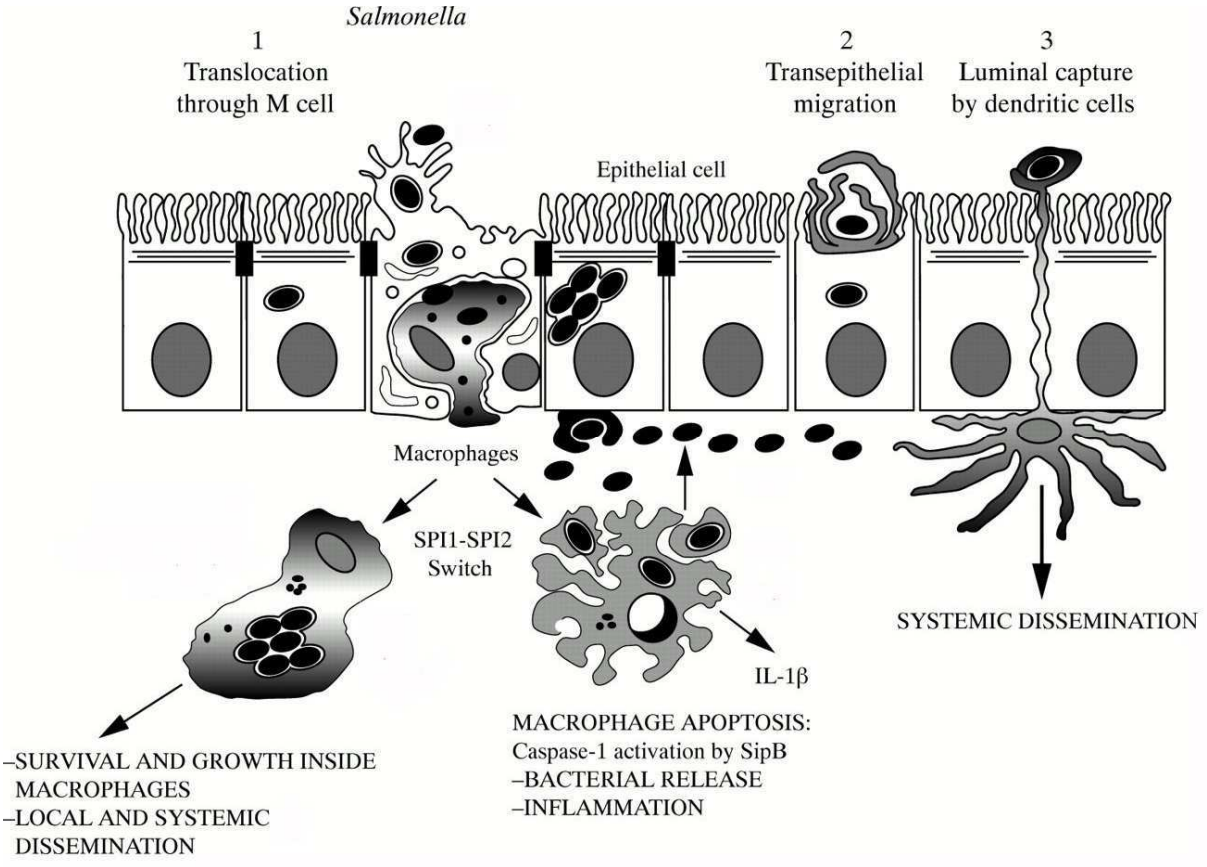



Fig. 1.7: *Salmonella* routes for crossing the intestinal barrier in the course of an oral infection. The single steps are described in the text. The figure is taken from Sansonetti, 2002.

The fact that *S. typhimurium* can easily be genetically modulated and manipulated can be favorable for tumor therapies. As described above, by deleting the gene for msbB, an altered LPS is present in the cell wall of this strain. This did not alter the tumor targeting behavior, but resulted in lower TNF-α stimulation and thus in lower toxicity (Low et al., 1999; Toso et al., 2002; Rosenberg et al., 2002).

As mentioned before, *S. typhimurium* theoretically meets the majority of the demands for an ideal anti-cancer bacterium. It can grow under aerobic and anaerobic conditions, it has the ability to survive in many of different host cells, including epithelial cells, macrophages and dendritic cells and it has the intrinsic property to preferentially invade solid tumors at tumor-liver ratios of 250:1 – 9000:1 (Pawelek et al., 1997). In contrast to obligate anaerobic bacteria like *Clostridia*, *S. typhimurium* does not only colonize big solid tumors with a huge hypoxic core. Rather they can even accumulate in micro-metastases after systemic administration (Yu et al., 2004). Nevertheless, several animal studies revealed that *S. typhimurium* although being able to live under aerobic conditions, still favors the necrotic tumor tissue, leaving the viable tumor cells intact. According to the study from Kasinskas and Forbes (2006), quiescent cancer cells produce compounds that specifically chemoattract the bacteria into the quiescent and necrotic tumor tissue. This results in the accumulation and proliferation of the bacteria inside the necrosis. Interestingly, although the initial attraction of *S. typhimurium* towards the necrotic area inside a solid tumor might partially be solved, the reason why they do not colonize the whole tumor and distribute evenly throughout the tumor tissue remained unclear thus far.

Concordantly, first clinical trials with an attenuated *S. typhimurium* in a Phase I study did not show the desired results. While most patients could tolerate the intravenous infection with the bacteria and some exhibited bacterial colonization of the tumor, only one patient responded to the treatment completely and was tumor free three months after infection. In all other patients, tumor growth was not retarded and the tumor biopsies of the examined tumors showed only focal colonization by bacteria. The authors mainly attributed the poor outcome of their study to the inability of the bacteria to fully colonize the tumors despite of the administration of a high dose (Toso et al., 2002; Heimann and Rosenberg, 2003).

#### 1.3.2.3 *Shigella flexneri*

A third potential candidate for bacteria-mediated tumor therapy is *Shigella flexneri* (*S. flexneri*), another member of the family of *Enterobacteriaceae* (Jennison and Verma, 2004). *S. flexneri* is a Gram-negative, facultative anaerobic, non-sporulating pathogen that is the cause for bacillary dysentery and shigellosis in man. Shigellosis is a bloody diarrhea that is caused by the invasion of the colonic and rectal mucosa by *S. flexneri*. Here, it leads to severe inflammation, which eventually results in the destruction of the mucosa (Sansonetti and Egile, 1998).

*S. flexneri* belongs to the genus *Shigella*, which comprises the four species *S. boydii*, *S. dysenteriae*, *S. sonnei* and *S. flexneri*, respectively. According to biochemical differences and variations in their O-antigens, which are the antigenic, polysaccharide (O) side chains of

the lipopolysaccharide (LPS) molecule in the intact bacterial cell of Gram-negative bacteria, these species are further divided into serotypes. Based on this classification scheme, the species *S. flexneri* can be divided into 13 different serotypes (Jennison and Verma, 2004).

*S. flexneri* is a highly infectious pathogen, as the uptake of 100 bacteria can be enough to cause shigellosis in adult humans (DuPont et al., 1989). This low infective dose is mainly due to *S. flexneri*'s ability to survive the low acidity in the host stomach by upregulating acid resistance genes like *rpoS,* the growth phase-dependent sigma factor sigma38 (Small et al., 1994).

During its natural course of infection, *S. flexneri* enters the body via the mucosal epithelium. Since *S. flexneri* is unable to invade epithelial cells through the apical route, this bacterium circumvents the apical entry by exploiting M cells in the FAE to enter into the colonic epithelium (Wassef et al., 1989). As a result, the majority of *S. flexneri* invade the epithelial cells through the basolateral pole of the colonic epithelial cells (Mounier et al., 1992). The reason for this detour via M cells and the resulting infection of epithelial cells via the basolateral pole still remains unclear. One possibility is that the mucin layer of colonic epithelial cells acts as a physical barrier to prevent the physical contact of the bacteria with the host cells, which is necessary for the bacterial entry into the cells. The second possibility is that the basolateral site of the epithelial cells displays molecules not present on the apical site, which function as receptors for bacterial cell entry.

*Shigellae* appear to enter the M cells via a process called "membrane ruffling" (Sansonetti and Phalipon, 1999). Although the translocation of *Shigella* through M cells has been observed and documented repeatedly (Sansonetti et al., 1996; Sansonetti and Phalipon, 1999), no specific adherence system that mediates the interaction between *Shigella* and the luminal side of M cells has been identified so far. As invasive *Shigella* translocate much more efficiently through M cells than non-invasive mutants, the invasive phenotype seems to play a major role in this process.

Upon internalization into an endocytic vacuole of the M cell, the bacteria are moved rapidly through the cell into the transepithelial pocket. Here, they are quickly phagocytosed by resident macrophages and dendritic cells. In order to obtain access to the basal side of the epithelial cells, *S. flexneri* cause apoptosis of the macrophages (Zychlinsky et al., 1992). Macrophages, which undergo *Shigella*-induced apoptosis, activate caspase-I, which eventually leads to the release of the two inflammatory cytokines. These are interleukin 1β (IL-1β) and interleukin 18 (IL-18) (Sansonetti et al., 2000). Both cytokines cause inflammation; the release of IL-1β additionally recruits polymorphonuclear cells (PMNs) to the site of infection (Zychlinsky et al., 1994). Once released into the sub-mucosa, *S. flexneri*

invade the epithelial cells through a macropinocytic process, in which bacteria-induced rearrangements of the cytoskeleton ("membrane ruffling") engulf the bacteria into a vacuole.

The uptake of *S. flexneri* into non-phagocytic cells like epithelial cell is in part conferred by products of the "invasion genes", which are encoded on a 210-230 kb virulence plasmid (Sansonetti et al., 1982; Hale and Keren, 1992; Schuch and Maurelli, 1997; Dorman and Porter, 1998). Many of these plasmid encoded virulence factors are located within a 31-kb "invasion region", which includes more than 30 genes (Maurelli et al., 1984; Sasakawa et al., 1988). Inside this invasion region, two loci are crucial for the uptake of *S. flexneri* into epithelial cells: the *ipa* locus and the *mxi-spa* locus. While the *ipa* locus encodes the "invasion plasmid antigens" IpaA, IpaB, IpaC and IpaD, which are the effectors of bacterial cell entry, the *mxi-spa* locus encodes the components of a type III secretion system, a needle-like structure that is used to deliver proteins from the bacterial cytoplasm to the cytoplasmic membrane or into the cytosol of the host cell (Tamano et al., 2000). The detailed mechanism by which the Ipa proteins cause bacterial cell entry has not completely been solved. Upon contact of the bacteria with the host cell, Ipa proteins are secreted. IpaB, IpaC and IpaD form a complex that has been shown to bind $\alpha 5\beta 1$ integrin that are present on the basolateral site of the epithelial cell (Watarai et al., 1996). Since $\beta 1$-inegrins interact with the actin cytoskeleton, these integrins might induce the observed "membrane ruffling". In addition, Tran et al. (1999) reported the activation of host cell Rho GTPases by IpaC, which triggers the actin polymerization and filopodial extensions in the vicinity of the bacteria.

A second receptor, CD44, is also expressed on the basolateral site of the epithelial cells and binds not only to hyaluronan, a component of the extracellular matrix, but also to an IpaB-IpaC complex (Skoudy et al., 1996a). The cytoplasmic domain of CD44 interacts with ezrin, a member of a protein family that crosslinks the plasma membrane with the actin cytoskeleton. Ezrin has been shown to be enriched in cellular protrusions that engulf invading *Shigella* (Skoudy et al., 1996b) and is thought to play an important role in the regulation of *Shigella* entry. In non-phagocytic cells, IpaC and IpaA seem to engineer the cytoskeletal rearrangements that are necessary for the uptake of the bacterium into the host cell (Tran et al., 1997; Bourdet-Sicard et al., 2000).

Inside the host cell cytoplasm *Shigellae* lyse the membrane-bound vacuole by the invasin IpaB and escape into the cytoplasm (High et al., 1992). Here, the bacterium is able to replicate and to directly spread from one epithelial cell to the other. The intracellular, actin-based motility and the cell-to-cell spread are directed by the outer membrane protein IcsA (Bernardini et al., 1989). IcsA is expressed on the surface of the bacteria, with the greatest concentration on the "old" pole of the bacterium. The unipolar localization of IcsA is essential

for intracellular movement of the bacterium and seems to be dependent on the structure of LPS (Goldberg et al., 1993; Sandlin et al., 1995; Charles et al., 2001).

The infection of the epithelial cells leads the activation of NF-κB, which ultimately results in the secretion of IL-8. Like IL-1β that is secreted by the infected macrophages IL-8 is a chemoattractant for neutrophils. As *Shigellae* are unable to escape the phagocytic vacuole of neutrophils, they are killed inside the phagosomes (Mandic-Mulec et al., 1997). Besides, leukocytes produce a host defense protein, neutrophil elastase (NE), which is capable to degrade *Shigella* virulence proteins within 10 minutes (Weinrauch et al., 2002). Thus, neutrophils obviously play an important role in controlling a *Shigella* infection. Unfortunately, neutrophils also migrate through epithelia, where they open the intercellular junctions between the epithelial cells and break the intact epithelial barrier. As result, *Shigellae* are now able to migrate directly to the basolateral site of the epithelial cells, sparing the detour via M cells and macrophages (Sansonetti et al., 1999; Philpott et al., 2000). A summary of the epithelial infection by *S. flexneri* is displayed in Figure 1.8.

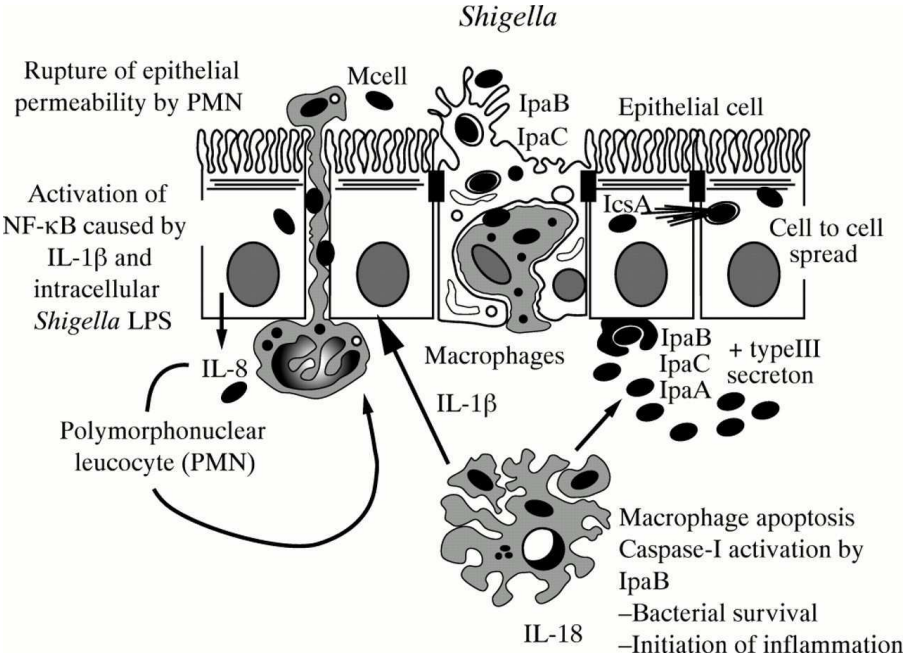



Fig. 1.8: *S. flexneri* routes for crossing the intestinal barrier during in the course of an oral infection. The single steps are described in the text. The figure is taken from Sansonetti, 2002.

In 2002, Lan and Reeves reported that *Shigella*, although it still stands as a genus with four species, belongs in fact to the heterogeneous species of *E. coli*. A comparative analysis of 36 housekeeping genes of *E. coli* K12 and *S. flexneri* 2a had revealed an average distance between the two species of 1.12%. This is within the range reported for *E. coli*. In addition, *Shigella* strains and EIEC strains share the presence of a large virulence plasmid and their

ability to induce their entry into epithelial cells and spread from cell to cell (Jin et al., 2002); (Parsot, 2005).

Being that closely related to *E. coli*, *S. flexneri* is not only a pathogen, but its attenuated forms can easily be used as vaccines, or as tools for gene transfer or protein delivery. In September 2000, Koprowski et al. reported the development of an attenuated *S. flexneri* 2a vaccine strain that expresses the colonization factor I antigen and mutant LT from ETEC. As intended, their prototype *Shigella*-ETEC hybrid vaccine could encode multiple ETEC antigens on a single plasmid in an attenuated *Shigella* vaccine strain and engender immune responses against the heterologous antigens, as well as against antigens of the vector strain.

Other vaccination strategies aim directly at the protection against Shigellosis, or more exact, at protection against multiple serotypes of *S. flexneri*. As immunity to *S. flexneri* is serotype-specific, the vaccination against one serotype will only provide protection against infections by the homologous serotype. The combination of several *S. flexneri* serotypes into a vaccine cocktail should make a cross-protection against most *S. flexneri* serotypes possible. Interestingly, a vaccine cocktail consisting of serotypes 2a and 3a could actually confer significant protection against challenges with the serotypes 1b, 2b, 5b and Y in guinea pigs (Noriega et al., 1999). Clinical trials with subunit *Shigella* vaccines revealed that proteosomes comprising native *S. flexneri* 2a LPS complexed to meningococcal outer membrane proteins were capable of generating a serotype-specific immune response in humans after intranasal administration (Fries et al., 2001). Similarly, *S. flexneri* LPS that had been attached to proteins and delivered parenterally was safe in humans and induced strong serum antibody responses (Cohen et al., 1996; Ashkenazi et al., 1999).

In summary, attenuated strains of *S. flexneri* have been widely used as direct vaccines against Shigellosis or as vectors for the delivery of DNA vaccines to mucosal tissues. Although many experiments and clinical trials have been carried out, which exploit *S. flexneri* as vaccine, *S. flexneri* have not been used as an anti-cancer bacterium so far. *S. flexneri* has not yet been reported to have intrinsic tumor targeting ability. Indeed *S. flexneri* did not specifically target into solid tumors in our own experiments. This might be attributed mainly to the fact that *S. flexneri* is a human pathogen that is not pathogenic in mice. Thus, most of the inoculum might be cleared almost immediately from the mice. Nevertheless *S. flexneri* strains were still able to migrate to and settle inside solid tumors after systemic administration of attenuated *S. flexneri* strains (unpublished data). However, one should keep *S. flexneri* in mind when looking for a suitable bacterium for anti-tumor therapy. As *S. flexneri* is that closely related to *E. coli*, it might be manipulated as easily and the same plasmids can be used for manipulating *E. coli* and *S. flexneri*. In addition, *S. flexneri* has repeatedly been shown to mediate gene

transfer into mammalian cells. This makes them an interesting option for transporting therapeutic molecules encoded as eukaryotic genes on plasmids into mammalian tumor cells.

Taken together, the investigation of the bacterial settlement inside solid tumors and the interaction of the bacteria with the tumor cells, as well as with different immune effector cells remains a major task in order to understand the preferential accumulation of facultative anaerobic bacteria like inside solid tumors. This knowledge would help to improve bacteria-mediated tumor therapies.

## 1.4 Bacteria-mediated tumor therapy

As described above, upon colonizing tumors, bacteria lead to increased necrosis and inhibition of tumor growth. The remaining viable cells at the tumor rim regrow thus resulting only in partial remission. Therefore, an important feature of anti-cancer bacteria is the possibility to exploit them as transport vehicles for therapeutic macromolecules. The selective accumulation of the bacteria results in transport of the therapeutic molecules into the solid tumor, exactly to the place, where they should fulfill their therapeutic effect.

Basically, there are three types of macromolecules that could be transported into solid tumors with bacteria as vehicles. (I), DNA, which is released into the cytosol of the infected mammalian tumor cells and is then translocated into the nucleus where the DNA is transcribed into mRNA. Thus, tumor cells express the therapeutic molecule themselves that should eventually lead to tumor destruction. (II), RNA that is delivered into the cytosol of the tumor cells, to be either translated into the encoded therapeutic molecule or to inhibit the expression of mammalian genes, e.g. by RNA interference. (III), therapeutic proteins, which are expressed by the bacteria. These proteins could remain in the bacteria, they could be secreted into the extracellular space in the tumor or they could be secreted into the cytosol of the tumor cell with the aid of appropriate secretion systems.

### 1.4.1 Bacteria-mediated gene transfer

One very exciting bacterial anti-cancer strategy is the transfer of eukaryotic expression plasmids via bacterial carriers into mammalian tumor cells. A schematic overview of bacteria-mediated gene-transfer is depicted in Figure 1.9.

Around 25 years ago, in 1980, Walter Schaffner (Schaffner, 1980) was the first to report direct transfer of plasmid-encoded Simian virus 40 (SV 40) DNA from *E. coli* into monkey cells. At that time, it was known that DNA can be transferred from one organism to the other within a kingdom, but gene transfer between prokaryotes and mammals was rather

unexpected. Thus far, the only well documented gene transfer between prokaryotes and eukaryotes had been the induction of crown gall tumors in higher plants by the non-invasive bacterium *Agrobacterium tumefaciens*. In the meantime, several attenuated, invasive bacterial strains including the Gram-negative bacteria *Salmonella typhimurium*, *Shigella flexneri*, invasive *Escherichia coli*, but also the Gram-positive species *Listeria monocytogenes* have been shown to efficiently mediate gene transfer into mammalian cells *in vitro* and *in vivo* (Sizemore et al., 1995; Courvalin et al., 1995; Darji et al., 1997; Hense et al., 2001; reviewed in Dietrich et al., 1999).

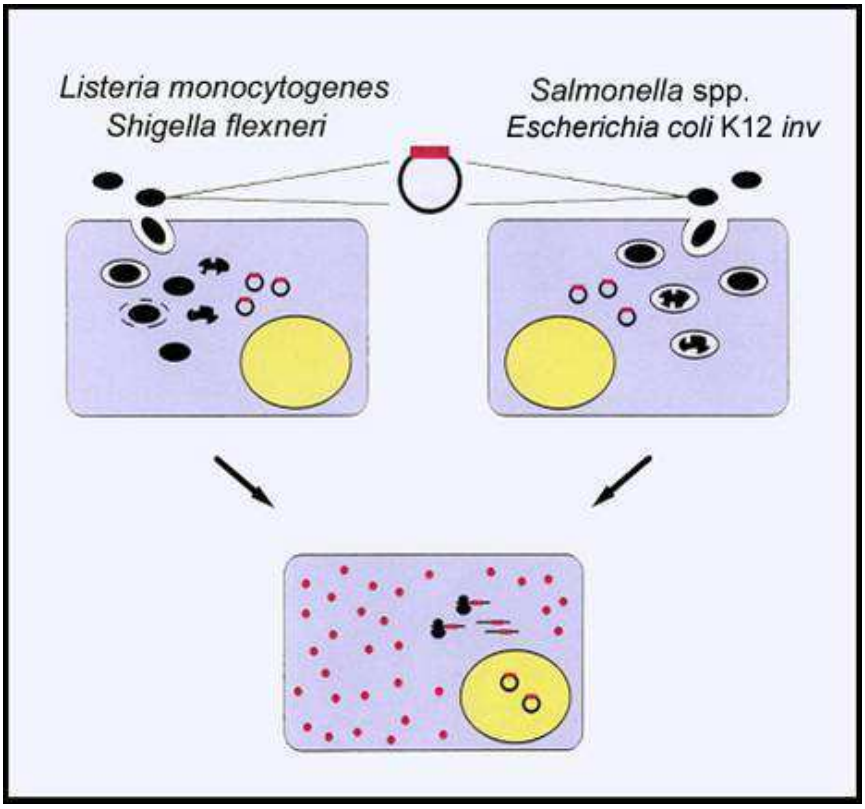



Fig. 1.9: Schematic overview of bacteria-mediated gene transfer into mammalian host cells. The basic principle of bacteria-mediated gene transfer is described in the text. The figure is taken from Weiss et al., 2001.

Even though the discovery of bacteria-mediated gene transfer into mammalian cells has been reported repeatedly, the exact mechanism of how the bacteria release their plasmid load and how the plasmid reaches the nucleus is still unknown. Basically there are two ways how bacteria can deliver their plasmid into the cytosol of the host cell. Invasive, intracellular bacteria like *S. flexneri* and *L. monocytogenes* are able to escape from the phagolysosome into the cytoplasm of the host cell. After escape into the cytosol these bacteria die either because of an attenuation that makes them auxotrophic for the production of essential proteins, nucleotides or cell wall components that cannot be supplied by the host cell (Sizemore et al., 1995; Courvalin et al., 1995), or they die by inducible autolytic systems or antibiotics that are

used to kill the bacteria inside the cytosol (Dietrich et al., 1998; Hense et al., 2001; Weiss and Krusch, 2001). The lysis of the bacteria results in the release of their plasmid load. In case of *L. monocytogenes* the bacterial escape out of the vacuole was shown to be essential for efficient gene transfer (Hense et al., 2001).

Interestingly, other bacteria like *S. typhimurium* or invasive *E. coli* that are unable to escape out of the phagosome and hence die within the vacuole, are still able to release their plasmid out of the vacuole into the cytosol (Darji et al., 1997; reviewed in Grillot-Courvalin et al., 1999). The mechanism of how plasmid DNA is transported into the cytosol remains unclear. In both cases the plasmid is transferred into the nucleus of the host cell, where it is transcribed into mRNA. The transport mechanism of the plasmid DNA into the host's nucleus is not yet solved. Finally, the mRNA is transported back into the cytoplasm and translated. The transgenic protein can be detected in the host cell.

Unfortunately, bacteria-mediated gene transfer per se is not very efficient as only a low percentage of infected eukaryotic cells will express the transgenic protein. In 2005 Zelmer et al. published a study in which they investigated the limiting step during bacteria-mediated gene transfer. According to their studies the bacterial plasmids are not simply released upon lysis, but remain associated with high molecular weight components. By further investigating the molecular mechanisms of plasmid transfer, bacterial gene transfer should become increasingly more efficient in the future.

When exploiting bacteria as vehicles for gene therapy the intended application is important. Not every bacterium that is capable of gene transfer is suitable for every application. Based on different host cell preferences, different bacteria naturally infect different host cells. *Salmonella* species for example transfect primarily murine and human macrophages and dendritic cells. This property most likely renders them highly suitable for genetic vaccinations (Gentschev et al., 1995; Darji et al., 1997; Catic et al., 1999; Grillot-Courvalin et al., 2002).

Contrary to *Salmonellae*, other bacteria like *L. monocytogenes*, invasive *E. coli* and *S. flexneri* have been shown to infect and transfect a broader range of cell lines, preferably epithelial cell lines. Even the transfer into primary cells has been reported (Grillot-Courvalin et al., 2002). Therefore, these bacteria might be more suitable for gene therapies other than vaccination, e.g. bacterial anti-cancer therapies. Furthermore, *L. monocytogenes* and *S. flexneri* are able to spread from cell to cell and might therefore spread inside the infected tissue (e.g. the tissue of a solid tumor) and transfect cells that are not accessible for other ways of gene therapy. Even *Salmonellae* have been shown to transfect different types of cell lines *in vitro* after the successful introduction of the poreforming toxin Listeriolysin O (LLO) from

*L. monocytogenes* into a *Salmonella* strain (Gentschev et al., 1995). However, this strain would still lack the advantage of cell to cell spreading.

Notwithstanding the low efficiency, bacterial transfection has been proven to be a useful tool in tumor therapy in mice. Lee et al. (2005) reported a successful transgene expression in murine melanomas after systemic administration of *Salmonella choleraesuis*, which was carrying a plasmid encoding thrombospondin-1. Thrombospondin-1 inhibits angiogenesis and modulates endothelial cell adhesion, motility and growth. Tumor-bearing mice that were treated with this *Salmonella* strain showed a significantly retarded tumor growth and a prolonged survival. The same group reported efficient gene transfer into murine melanoma and bladder tumors by *S. choleraesuis* carrying an endostatin expression vector, which exerts tumoricidal and anti-angiogenic activities (Lee et al., 2004).

Other approaches aim at bacteria-mediated DNA vaccination against specific tumor antigens (Weth et al., 2001; Niethammer et al., 2001; Xiang et al., 2001; Pertl et al., 2003; Souders et al., 2006) and at the additional stimulation of the anti-tumoral immune response by coexpression of cytokines (Luo, 2004) or CD40-ligand (Xiang et al., 2001).

All these reports highlight a noticeable effect that is caused by bacterial gene transfer. However, there is still one obvious limitation associated with such studies, namely the instability of the transfectants used. It is known, that high copy plasmids are quickly lost by the bacterial vehicle *in vitro* and especially *in vivo*, as a high number of plasmids represent a burden for the bacterium (Togna et al., 1993; Glick, 1995). In 2005 Bauer et al. circumvented this problem by designing low copy plasmids, which were dramatically more stable both *in vitro* and *in vivo* and resulted in improved immune responses.

Recently Giacalone et al. (2006) presented an interesting new approach, in which they used protoplasted, achromosomal bacterial minicells to transfer plasmid DNA into eukaryotic Cos-7 cells. Minicells are small, achromosomal vehicles that are produced through ectopic septation during cell division and contain all molecular components of the parent cell minus the chromosome (Khachatourians et al., 1973). The use of bacterial mini cells that mainly consist of the bacterial envelope and the plasmid that has to be transferred might be a future direction of bacteria-mediated gene transfer. Minicells are almost as easy to manipulate as bacteria, they are smaller and their plasmid load is stable. They show no toxicity to cultured cell lines (Giacalone et al., 2006) and the co-transfer of DNA of the bacterial chromosome that might generate a problem is avoided.

Taken together, the use of bacteria as transfer vehicles for plasmid DNA still remains a promising tool for successful cancer therapy in the future.

### 1.4.2 Bacteria-mediated RNA transfer

A novel approach for bacteria-mediated tumor therapy is the use of tumor-targeting bacteria as carriers for RNA. Thus far, two different applications for bacterial RNA transfer into eukaryotic cells have been reported: the delivery of functional mRNA (Schoen et al., 2005; Loeffler et al., 2006) and the delivery of short hairpin RNA (shRNA) that elicits RNA interference in mammals (Xiang et al., 2006). Schoen et al. (2005) used a self-destructing *L. monocytogenes* strain to efficiently deliver translation-competent mRNA into the cytosol of epithelial cells, macrophages and dendritic cells *in vitro*. The method was further improved by Loeffler et al. (2006), who used the same bacteria *in vivo*, where they successfully delivered the model antigen ovalbumin (OVA). This resulted in the presentation of OVA in the context of major histocompatibility complex (MHC) class I molecules and to induction of a specific CD8 T-cell response. The way from the described vaccination strategies to the use of bacteria to transfer mRNA in anti-cancer therapies is rather obvious.

In contrast, Xiang et al. (2006) used non-pathogenic, invasive *E. coli* to transcribe shRNAs against catenin β-1 from a plasmid, which induced significant gene silencing in the intestinal epithelium and in human colon cancer xenografts in mice. This approach has the potential to be further developed to clinically compatible RNAi-based anti-cancer therapies.

Both forms of bacterial RNA transfer can be seen as potential alternatives to bacteria-mediated DNA transfer as they have the advantage to lack the limiting step that impairs bacterial DNA transfer, i.e. the transport of the plasmid into the nucleus. On the other hand, only transient effects can be obtained with this approach.

### 1.4.3 Bacteria-mediated delivery of therapeutic proteins

A second alternative to bacteria-mediated gene transfer is to utilize tumor-targeting bacteria for the delivery of proteins. In fact, the delivery of therapeutic proteins by bacteria will in all probability become the dominant first generation of applications of bacterial therapies. Due to its importance, a detailed description is given here.

As mentioned above, there are three different mechanisms via which bacteria can deliver therapeutic proteins: The proteins can remain in the bacteria, they can be secreted into the extracellular space in the tumor, or, they can be secreted into the cytosol of the tumor cell. The most difficult objective is to find a suitable expression system that is optimal for the intended application.

An example for a therapeutic protein that remains inside the bacteria is CD. As described before, a plasmid with the gene that expresses the *E. coli* enzyme CD has successfully been

introduced into different tumor-targeting bacteria, including *Clostridia*, *Bifidobacteria* and *Salmonellae* (Fox et al., 1996; King et al., 2002; Liu et al., 2002; Fujimori, 2006; Sasaki et al., 2006). By expressing functional CD intracellularly, the bacteria can transform the non-toxic prodrug 5-FC into the cytotoxic drug 5-FU (compare Fig. 1.4). An alternative enzyme would be nitroreductase, which converts the prodrug CB1954 to a highly toxic bifunctional alkylating agent. This enzyme has been used in animal experiments with *Clostridia* already (Minton et al., 1995).

A simple possibility to release intracellular proteins from the bacteria into the extracellular space exists by the induction of bacterial lysis. Recently, the lytic gene E from bacteriophage ΦX174 could be shown to be expressed in tumor-targeting *Salmonellae* upon induction of an L-arabinose controlled promoter. Additionally, this promoter could successfully be induced inside tumor-residing *Salmonellae* (Loessner et al., 2007). Other approaches that aim at the release of intracellular proteins into the extracellular space utilize particular metabolic attenuations. The release of intracellular macromolecules could be shown for a *Salmonella* mutant that is defective in asd, an essential enzyme in cell wall synthesis (Loessner et al., 2006). However, this approach has many limitations like the uncontrolled release of additional bacterial products.

In contrast, the exploitation of special bacterial secretion systems facilitates a controlled release of proteins from bacteria. Secretion systems are available that can be used to deliver proteins either into the extracellular space or into the cytosol of the adjacent cell.

Many Gram-negative bacteria use the so-called type I secretion systems (type I SS) to translocate proteins like pore-forming toxins, proteases, lipases or S-layer proteins across their inner and outer membranes into the extracellular surroundings. The prototypical and best characterized type I SS is the *E. coli* α-hemolysin (Hly) secretion system. A topological model of the hemolysin secretion system is depicted in Fig. 1.10.

Type I secretion systems characteristically contain only three different transport components, HlyB, HlyD and TolC. HlyB and HlyD are located in the inner membrane (IM) and are specific for the passenger protein that is to be translocated across the membrane, while TolC forms a pore in the outer membrane (OM) (reviewed in Gentschev et al., 2002). During the translocation process, these three proteins form a tunnel that links the IM and the OM. As a result, the passenger proteins are secreted directly into the extracellular medium. Type I SS recognize a secretion signal (HlyA), which is located at the carboxyl terminus of the secreted proteins and is usually not cleaved off after secretion.

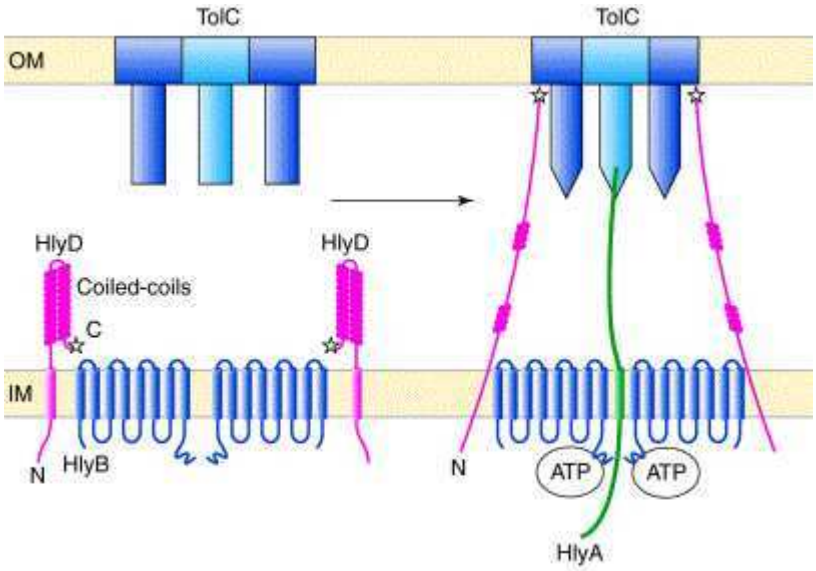



Fig. 1.10: Topological model of hemolysin secretion. In response to HlyA engagement, a HlyD trimer interacts with the trimeric TolC protein to form a continuous transperiplasmic export channel. The Figure is taken from Gentschev et al., 2002.

Some of the gene clusters encoding such secretion systems are already located on plasmids and are therefore easy to be modified genetically, a sincere advantage of type I SS (Binet et al., 1997). In addition, the plasmids carrying the Hly system of *E. coli* have been shown to replicate stably in other Gram-negative bacteria, including *Vibrio cholera* and various species of *Salmonella* and *Shigella* (Spreng et al., 1999). This secretion system also appears to support the secretion of heterologous proteins independent of their size (Hahn and von Specht, 2003). Thus, it can be modified to express and secrete proteins of potential therapeutic value in the course of bacteria-mediated anti-cancer therapy.

However, the Hly secretion system also has its limitations. The amount of recombinant protein that is secreted under *in vitro* conditions is rather low (Li et al., 2000; Li et al., 2002). Besides, the secretion is tightly coupled to bacterial growth and the accumulation of HlyA fusion proteins occurs only in the early and mid-exponential growth phase (Felmlee et al., 1985; Li et al., 2000). The impact of bacterial growth inside solid tumors on the secretion of such fusion proteins is yet unknown, as the knowledge about growth phases of bacteria inside solid tumors is scarce.

The secretion of HlyA-fusion proteins was further found to be oxygen dependent. As small culture volumes resulted in higher final protein concentrations (Hahn and von Specht, 2003), aeration seemed to negatively affect the secretion of HlyA fusion proteins. Ironically, what is detrimental *in vitro* might be beneficial for *in vivo* applications. If this observation is confirmed in *in vivo* studies, it might become an advantage for bacterial anti-cancer therapies

in solid tumors, which are known to have large necrotic areas, in which the bacteria preferentially accumulate.

Another limitation of HlyA secretion systems is the relatively large secretion signal sequence, which consists of 60 amino acids and is not removed after translocation. This signal sequence might influence the correct folding of the recombinant protein and lead to the secretion of non-functional proteins. In addition, Hly-mediated secretion is greatly impaired for proteins that contain extended β-sheet regions (Hahn and von Specht, 2003).

However, Hahn et al. (1998) were able to secrete functional, bioactive human interleukin 6 (hIL-6) via this secretion system with *S. typhimurium*. Several years later, Li et al. (2003b) reported an optimized hIL-6 secreting *S. typhimurium* strain that could increase systemic IgG and IgA responses against bacterial LPS upon oral administration of vaccine strains *in vivo*. In the meantime Hly secretion systems have been used to successfully secrete several different vaccine antigens (Gentschev et al., 1994; Hess et al., 1996; Gentschev et al., 1998; Hess et al., 2000; Spreng et al., 2000) mucosal adjuvant proteins like the subunits of choleratoxin (CT) and the tetanus toxin fragment C (Harokopakis et al., 1997; Lee et al., 2000) as well as cytokines including the interleukins IL-4, IL-5 and IL-6 (Denich et al., 1993; Dunstan et al., 1996; Whittle et al., 1997).

In contrast to the hemolysin secretion system, bacterial secretion systems exist, which transport the protein of interest directly into the cytosol of the tumor cell. One such system is the type III secretion system (type III SS). The name “type III secretion system” refers to a secretion pathway, which is common to the flagellae of eubacteria and the injectisomes of some Gram-negative bacteria (reviewed in Journet et al., 2005). While flagellar secretion results in the transport of the protein into the extracellular space, injectisomes release the protein into the cytosol of the cell. A model of an injectisome is shown in Fig. 1.11.

The flagellum is a motility organelle that – besides its function as a rotary motor for bacterial movement – contains a built-in secretion apparatus for the export of the hook and filament components (Macnab, 2003). In 2005 Majander et al. were able to explore the flagellar secretion apparatus of *E. coli* for extracellular secretion of several polypeptides and eukaryotic GFP at high concentrations. This secretion system was claimed to be only dependent on the 5’ untranslated region (5’UTR) plus an N-terminal secretion signal of 20 amino acids resulting in a significantly smaller fusion protein compared to HlyA-fusion proteins. However, contrary to the findings of Majander et al. (2005), Gal et al. (2006) and Vegh et al. (2006) reported a 5’UTR independent protein secretion in *S. typhimurium* and a flagellum specific secretion signal confined to a 22-amino acid sequence in the N-terminal region of *Salmonella* flagellin.

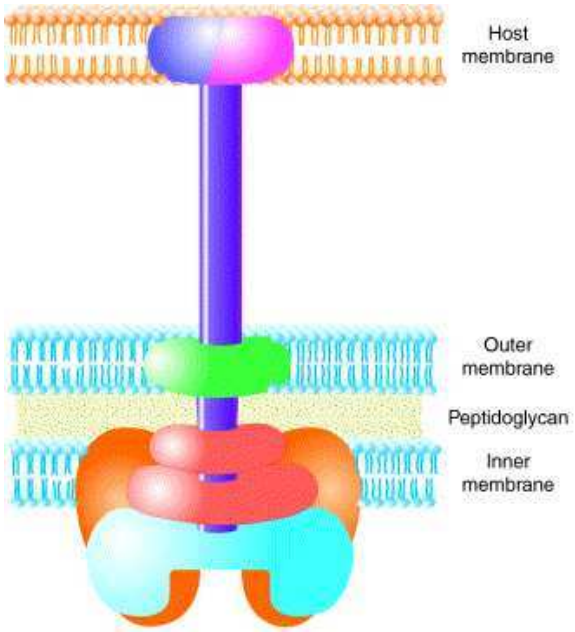



Fig. 1.11: Representation of a type III secretion system (injectisome) modeled from isolated needle structures observed by electron microscopy and genetic and biochemical data. The figure is taken from Thomas and Brett, 2003.

Injectisomes are needle-like structures that allow the bacteria to deliver proteins across the two bacterial membranes and the eukaryotic membrane into the host cell's cytosol. Although similar between different species, every bacterial species has a slightly different type III SS with different secretion signals in each species. Usually the secretion systems are not transferable from one species to another and thus have to be designed specifically for the favored species for each application.

Up to now, type III SS have mainly been used to secrete vaccines (Russmann et al., 1998; Russmann et al., 2002). Nishikawa et al. (2006) and Panthel et al. (2006) were even able to deliver anti-tumor antigens via the *Salmonella typhimurium* type III SS. In the first case, this resulted in $CD8^+$ T cell induced tumor regression. In the second case, prophylactic cytotoxic effector and memory $CD8^+$ T cell responses against a murine fibrosarcoma were induced.

In 2002 Russmann et al. reported the successful secretion of proteins using the InvI/InvJ type III secretion system of *S. typhimurium*. According to their studies, as little as 7 amino acids at the N-terminus of the secretion sequence were necessary and sufficient to correctly secrete the fusion protein.

Theoretically all kinds of proteins can be secreted via a type III SS. Besides their use for the secretion of antigens, they should also be suitable to release therapeutic molecules for anti-cancer therapies. Many type III systems seem to be more efficient regarding protein concentration and length of signal peptide compared to type I SS. On the other hand, type III

SS are very complex and consist of at least twenty different proteins, which render them difficult to genetically manipulate. Besides, not all proteins are secreted efficiently. Disulfide bonds cannot be built correctly. They cannot be transferred from one species to another and they still result in the release of fusion proteins that might be incorrectly folded (reviewed in Journet et al., 2005).

Type IV secretion systems (type IV SS) are similar to type III SS in assembly, function and in their limitations. These secretion systems are involved in horizontal gene transfer to other bacteria and eukaryotic cells. The prototypical type IV SS is the *Agrobacterium tumefaciens* T-DNA (transfer DNA) transfer machinery, which delivers the T-DNA into plant cells and results in the development of crown-gall tumors. Other bacteria, such as *Helicobacter pylori*, use it for the injection of protein virulence factors into the host's cytosol. However, this system has not been used for the transport of therapeutic proteins yet (reviewed in Backert and Meyer, 2006).

An alternative type of secretion system that might be suitable for the application in bacterial anti-cancer therapies are autotransporters of the type V secretion systems (type V SS). Autotransporters are proteins that contain all of the information required for traversing the bacterial membrane system and routing to the bacterial cell surface. This indicates that they are characterized by an outer membrane translocator module representing the C-terminal domain of the transported protein itself. Different to the above mentioned secretion systems, the signal peptide can be cleaved off after transport, provided that an appropriate motif is included. Thus, the folding of the heterologous protein is not impaired by an additional signal peptide (Konieczny et al., 2001). One prominent member of the autotransporter family is the AIDA autotransporter, which is a bacterial adhesin associated with some diarrheagenic *E. coli* (Benz and Schmidt, 1992; Sherlock et al., 2004). The AIDA system consists of a translocator subunit that forms a β-barrel porin in the outer membrane through which the adhesin moiety gains access to the surface (Maurer et al., 1999). Up to now, autotransporters have mainly been used for vaccination strategies (Rizos et al., 2003; Kramer et al., 2003; Casali, 2003). Like the other described secretion systems they do have the potential for a future use as secretion system of therapeutic molecules in bacteria-mediated anti-cancer therapy.

Additional secretion systems exist for various applications, e.g. for the secretion of recombinant antibodies. While Gram-negative bacteria like *E. coli* secrete the proteins mostly into the periplasm (reviewed in Ward, 1993) and thus have to be lysed in order to release the protein into the extracellular space, Jordan et al. (2007) exploited the Gram-positive bacterium *Bacillus megaterium* for the production of recombinant antibody fragments, which were successfully secreted into the growth medium. Although a tumor targeting ability of this

bacterium has not been tested yet, it might have chances as therapeutic vehicle in anti-cancer therapies.

In general, all described secretion systems could be simply applied for vaccination strategies, in which functionally folded proteins are not essential. In contrast, the secretion of biologically active proteins, such as cytokines or cytotoxins, requires correct folding and is therefore more prone to failure. As a prediction of the most adequate secretion system that is working for the given protein is not possible, different secretion systems will have to be tested systematically in each case.

## 1.5 Bacterial interactions with the immune system in tumor therapies

The immune system of vertebrates is a remarkable adaptive system of defense. It has evolved to protect the host from invasive microorganisms and includes an extraordinary huge number and variety of cells and molecules that are able to specifically detect and eliminate foreign invaders. All these cells and molecules cooperate in a unique, dynamic network, whose complexity is still far from being understood (reviewed in Delves and Roitt, 2000a; Delves and Roitt, 2000b).

In general, one can distinguish the immune reactions that are initiated upon contact with a pathogen into adaptive and innate immunity. The adaptive immunity is only activated if the invading microorganisms have not been eliminated by the innate immune system already. It is based upon the production of antibodies by B cells and effector functions of T cells. The adaptive immune response often leads to a lifelong immunity against infections by the same microorganism. Thus, the activation of the adaptive response during bacteria-mediated tumor therapy might prevent repeated administration of the therapeutic bacteria (reviewed in Medzhitov and Janeway, Jr., 1998).

As the adaptive immune response is activated at later times during an infection and only in cases where the innate immune system was not able to eliminate the intruding bacteria, it should only play a minor role at the early times of bacteria-mediated tumor therapy. Thus, to investigate the immediate impacts of bacterial colonization of solid tumors on the immune system, the major focus should lie on the immediate innate immune response, i.e. the first line of defense. Depending on the type of bacterial tumor therapy, the natural activation of the immune system by the bacterial carrier might not necessarily be beneficial. Some tumor therapies might actually require an inducible suppression of the host’s immune system or the stimulation and shaping of the immune answer of the host according to the therapeutic needs.

### 1.5.1 First line of defense

Innate immunity comprises four different defense mechanisms, which protect the host from bacterial, viral, fungal and parasite infections. These defense mechanisms comprise physical barriers like the skin or the surface of mucous membranes that keep pathogens from invading the body mechanically, physiological barriers, phagocytic or endocytic barriers and inflammatory barriers (reviewed in Medzhitov and Janeway, Jr., 2000; Janeway, Jr. and Medzhitov, 2002). For intended infections in the course of bacteria-mediated tumor therapies physical barriers should not play a role, as the bacteria are injected intravenously and naturally find their way into the tumor tissue or are even injected directly into the tumor. The same is true for physiological barriers. As bacterial tumor therapy vectors are applied according to the therapeutic needs, physiological barriers like the low pH of the stomach or the mucus and digestive enzymes in the upper intestinal tract, amongst others, are simply circumvented. In addition, therapeutic bacteria are especially chosen to find optimal growth conditions inside the tissue of solid tumors and are therefore well adapted to their host and to their target tissue in particular. Thus, barriers that do influence bacterial tumor therapy and might even direct the outcome of the therapy are inflammatory barriers and phagocytic or endocytic barriers.

If a pathogen has breached the physical and physiological barriers and escaped into the host's body during a natural occurring infection, the innate immune system of the host tries to eliminate the intruder immediately or to retain the pathogen locally.

Activation of innate defense mechanisms are due to various pathogen associated molecular patterns (PAMPs) that distinguish microorganisms from multicellular organisms. Correspondingly, the innate immune system has evolved "pattern recognition receptors". Among these microbial patterns that are specific for bacteria are peptidoglycan, lipopolysaccharide and lipoteichoic acid (Kimbrell and Beutler, 2001).

The activation of the pattern recognition receptors, which include the entire toll like receptor family (TLRs) amongst others, results in a cellular signaling cascade that ultimately leads to the secretion of inflammatory and chemoattractant proteins like cytokines and chemokines (Penate and Pena, 2001). This causes an inflammatory reaction that is characterized by swelling, redness, heat and pain (Bloom and Ahmed, 1998) and leads to an enlargement of the local blood vessels. As a consequence, the local blood flow is increased while the velocity of the blood flow decreases and the tissue becomes red and warm. The infiltration of professional phagocytes like macrophages and neutrophilic granulocytes is initiated by the expression of adhesion molecules on the surface of the endothelial lining. Simultaneously the permeability of the blood vessels is increased as the joining of the endothelial cells that line

the blood vessels loosens. Monocytes, NK cells and granulocytes that patrol the blood to detect pathogens are entering into the infected tissue accompanied by liquid and antimicrobial proteins. This causes the swelling and pain. The phagocytes like neutrophilic granulocytes and macrophages that accumulate inside the infected tissue will eventually take up and destroy the pathogenic invaders by a process called phagocytosis (reviewed in Aderem and Underhill, 1999).

### 1.5.2 Neutrophilic granulocytes

Neutrophilic granulocytes, so-called neutrophils, belong to the class of polymorphonuclear leukocytes, whose name is based on the multilobulated shape of their nuclei. Leukocytes in general comprise macrophages, granulocytes and dendritic cells. The group of granulocytes can further be divided into basophilic, eosinophilic and neutrophilic granulocytes or neutrophils. The latter represent 50-60% of the total circulating leukocytes and are the first cells to be recruited to the site of infection during an inflammatory reaction. Being active phagocytes, neutrophils target bacteria, viruses, fungi, protozoa, virally infected cells and tumor cells.

Like other cells of the immune system, neutrophils are derived from pluripotent stem cells in the bone marrow (Baum et al., 1992). During granulopoiesis the hematopoietic stem cells give rise to unipotent myeloblasts, which subsequently differentiate via five further morphological stages into segmented neutrophils (Cline and Golde, 1975). The development in the bone marrow takes about two weeks. During this time the cells sequentially acquire the receptors and proteins that are needed for their role in the first line of defense. Once they are mature, they are released into the bloodstream, where they circulate with a half life of 6-8 hours before they migrate into tissues. Here they can survive for 1-2 days (Kobayashi et al., 2005).

Neutrophils are actively recruited to the sites of infection in response to host- or pathogen-derived chemotactic factors such as the chemokine IL-8 in humans (Godaly et al., 1997) and its murine homologue MIP-2 (macrophage-inflammatory protein-2) (Tekamp-Olson et al., 1990) or bacterial products like N-formylated peptides. Many of these chemoattractants can prime neutrophils to enhance their function, which improves host defense. They might be primed for enhanced adhesion, phagocytosis and production of reactive oxygen species (ROS), cytokine secretion, leukothriene synthesis, degranulation and bactericidal activities. Neutrophil priming agents include host-derived cytokines, chemokines, growth factors and lipid-derived signaling molecules, but also all kinds of microbial products, cell-cell contact and the adhesion to cell receptors (reviewed in Kobayashi et al., 2005). Priming of neutrophils

mobilizes secretory vesicles and induces the secretion of cytokines to promote an efficient clearance of the invading pathogen.

At the site of infection neutrophils bind and ingest the invading microbes via phagocytosis. The binding of PAMPs to pathogen-recognition receptors like TLRs and CD14 activates signaling pathways that lead to the above mentioned effects of priming.

Microorganisms could be opsonized, i.e. covered with activated serum complement components or specific antibodies. Such microbes are recognized and bound via complement receptors and Fc receptors that bind to the constant part (Fc region) of the antibodies. This process drives the mechanism of phagocytosis in which the microorganisms are internalized and sequestered within a phagosome.

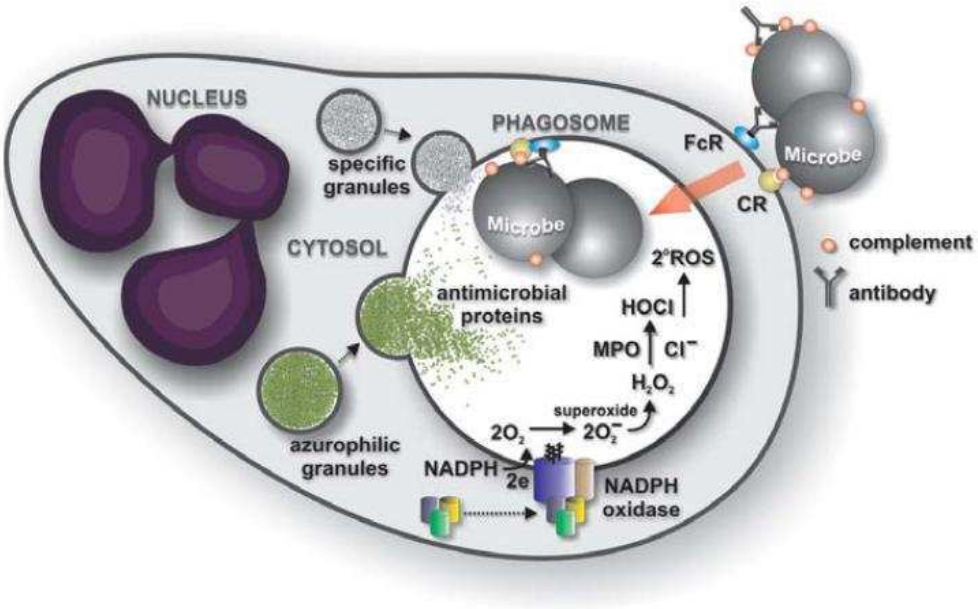



Fig. 1.12: Neutrophil phagocytosis and activation of microbicidal systems. The exact mechanisms are described in the text. The figure is taken from Kobayashi et al., 2005.

Inside the phagosome the microbes are killed by two independent mechanisms, an oxygen-dependent and an oxygen-independent mechanism. In the first mechanism, neutrophils undergo a so-called “respiratory burst” during which the NADPH oxidase complex assembles at the phagosomal membrane and produces $O2^{-}$. This is converted into ROS such as hydrogen peroxide, hypochlorous acid, hydroxyl radical and chloramines, all of which are potent antimicrobial agents (Hampton et al., 1998; Chapman et al., 2002).

In the oxygen-independent mechanism, specific cytoplasmic granules that contain anti-microbial peptides and proteins like α-defensins, cathepsins, proteinase-3, elastase, azurocidin and lysozyme, fuse with the bacteria-containing phagosomes (Flodgaard, 2003). The content

of the granules is released and consequently the phagosome is enriched with microbicidal proteins and peptides that degrade bacterial proteins or disrupt the anionic bacterial surfaces (Borregaard and Cowland, 1997). These described mechanisms are illustrated in Figure 1.12.

In 2003 Reeves et al. proposed a convincing concept that combines the two mechanisms. If ROS recruit $K^+$ ions into the phagolysosome, the potassium ions can further allow cationic proteases of the azurophilic granules to convert from a highly organized intracellular structure in a soluble form. This will result in the fusion of the granules with the bacteria-containing phagosomes and the bacteria are destroyed by the released microbicidal proteins. (Reeves et al., 2003)

Recently Brinkmann et al. (2004) and Fuchs et al. (2007) reported a novel mechanism whereby neutrophils can kill bacteria. By releasing granule proteins and chromatin into the extracellular medium, neutrophils generate extracellular fibers or “neutrophil extracellular traps” (NETs) that bind Gram-positive and -negative bacteria. These NETs degrade virulence factors and kill bacteria extracellularly. Besides the antimicrobial properties, NETs might serve as physical barriers that prevent the spreading of the microorganisms throughout the body. An electron microscopical image of such NET-generating neutrophils with *Shigellae* entrapped in the NETs can be seen in Fig. 1.13.

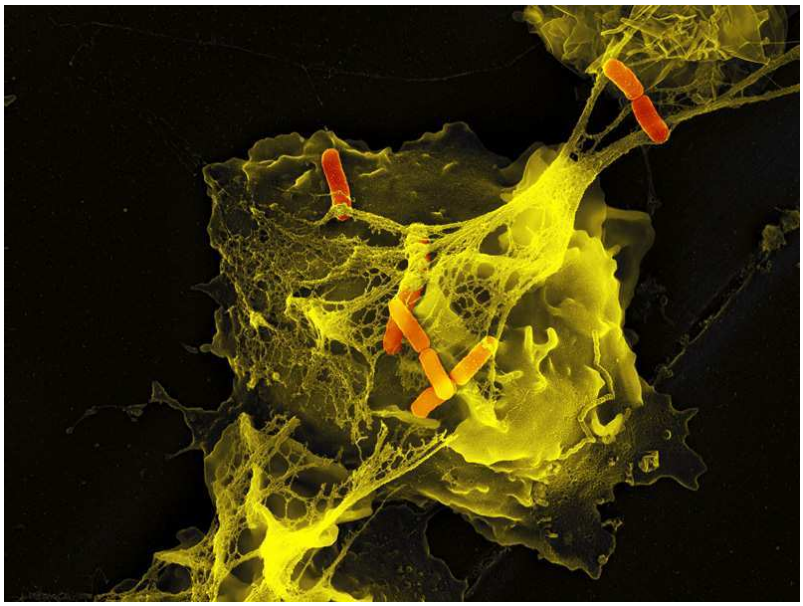


Fig. 1.13: Electron microscopy of a NET-generating neutrophil. *Shigellae* are entrapped by the neutrophils. The figure is taken from Zychlinsky, 2006.

As counterattack to strategies of the immune system, bacteria have developed own strategies to outflank the defense mechanisms of this system. These strategies include avoidance of neutrophil contact, the prevention of phagocytosis, the survival within the neutrophils, the

killing of neutrophils and the avoidance of being killed in neutrophil NETs (Urban et al., 2006).

Neutrophils have been repeatedly reported to infiltrate bacteria-infected solid tumors in the course of a bacteria-mediated anti-tumor therapy. Once they have migrated into the hypoxic/necrotic regions of the tumor, their functions appear to be inhibited (Bjerknes et al., 1990). The same has been shown for other immune cells (macrophages and T cells) under hypoxic conditions (Siegert et al., 1999; Robbins et al., 2005).

Nevertheless, the above mentioned interactions between neutrophils and bacteria could play a major role in tumor therapy and influence the outcome of the anti-cancer therapy in a beneficial or detrimental way.

### 1.5.3 Macrophages and the stimulation of the specific immune system

The second type of leukocytes are macrophages. Macrophages are an extremely heterogeneous population of cells. They develop in bone marrow and migrate through the blood as monocytes. In the tissue they develop into mature macrophages, which can become extremely long-lived. Similar to neutrophils, macrophages are actively recruited to the site of infection upon stimulation with chemotactic factors. Being professional phagocytes, macrophages can phagocytose bacteria, viruses or proteins upon contact with the foreign antigen. Having bound the antigen with one of the different receptors such as mannose-, scavenger- or Fc-receptor on the surface of the phagocyte, phagocytosis is initiated (Pontow et al., 1992; Ravetch, 1997).

Macrophages are activated by phagocytosis. The activation can be enhanced by cytokines that are released by other immune cells, e.g. activated $T_H$-cells (Aderem and Underhill, 1999). Activated macrophages are more effective in killing and eliminating pathogens than resting macrophages. They are able to secrete a number of cytotoxic proteins that kill virus-infected cells, tumor cells or intracellular bacteria (Gordon, 2003).

Characteristic for activated macrophages is the upregulation of MHC (major histocompatibility complex) class II molecules on their surface. These molecules present antigens to $T_H$-cells of the specific immune system. In case of a bacterial infection these antigens are polypeptides that resulted from the lysis of the phagocytosed bacterium. Thus, macrophages are not only part of the host's first line of defense, but they can also induce secondary immune responses by stimulating the specific immune system (Margulies, 1997).

The role of macrophages in bacteria-mediated anti-tumor therapy is yet unknown. However, an influence of macrophages on bacterial dissemination and on the recruitment of different immune cells to the tumor upon bacterial infection cannot be excluded.

DCs, the third member of leukocytes, were long considered to be macrophages. Now it is clear that they are an independent cell lineage. They are essential for the induction of a specific immune response, but at the present state of knowledge they are irrelevant for bacterial tumor therapy.

## 1.6 Aim of this work

Several obligate and facultative anaerobic bacterial strains are known to preferentially accumulate and proliferate inside solid tumors when given systemically (Yu et al., 2004). Their ability to naturally target to and accumulate in solid tumors suggests their use as transport vehicles that carry therapeutic molecules directly into solid tumors. Thereby it could be possible to limit side effects that are caused by non-directed, systemic treatments by commonly used anti-cancer therapeutics.

At present only little is known about the mechanisms that lead to bacterial settlement in solid tumors and about bacteria-host interactions inside the tumor. In addition, the majority of tumor-targeting bacteria show poor dissemination inside solid tumors as they accumulate exclusively inside large necrotic areas, and spare a rim of viable tumor cells (Vassaux et al., 2006). The reason for the limited dissemination is still little understood.

The present work deals with several aspects of early bacterial tumor colonization and the impacts of bacterial settlement inside solid tumors. It focuses on the use of three different facultative anaerobic bacteria, namely *S. flexneri*, *S. typhimurium* and *E. coli*, as vehicles for bacteria-mediated tumor therapy. The investigations of this work should reveal new informations about tumor composition and the infiltration of primary immune cells, about the interactions of the bacteria with these immune cells and about influences of tumor composition and tumor microenvironment on the bacteria and their behavior.

One aspect of this work was to establish an *S. flexneri* strain, as an *in vivo* system for bacteria-mediated gene transfer into solid tumors. As Gram-negative bacteria are able to use the mammalian CMV promoter for gene expression (Goussard et al., 2003) an intron should be integrated into a reporter gene. This should allow to ultimately distinguish eukaryotic from bacterial gene expression and to manifest the proof of principle of *in vivo* bacterial gene transfer as a means for tumor therapy.

A second aspect of this work was the characterization of early bacterial settlement inside solid tumors. This included the characteristic dissemination of the above mentioned facultative anaerobes in a model tumor and the impact of bacterial colonization inside solid tumors on the infiltration and distribution of immune cells. Histological analysis of bacteria-colonized

model tumors at early times post infection, ranging from 30 min p.i. to 2 days p.i., should clarify how the bacteria escape from the blood stream into the solid tumor and how they start to colonize and disseminate throughout the solid tumor. Such analyses should further give information about the chronology of bacterial tumor colonization after systemic infection and the associated effects on the host's immune system. The results obtained from histology should be confirmed and extended with electron microscopy of infected model tumors. Especially the question whether the bacteria reside intra- or extracellularly should be answered here.

These investigations led to the final issue of the present work. As the majority of tumor-targeting bacteria show poor dissemination inside solid tumors, the improvement of bacterial spreading in solid tumors to vital parts of the tumor should be addressed. This should result in a homogeneous distribution of the bacteria and of therapeutic factors that are to be carried by the tumor-targeting bacteria.

# 2 Material and Methods

## 2.1 Material

### 2.1.1 Mouse strain

6 week old, female BALB/c mice (Harlan, Borchen, Germany) were used for all animal experiments.

### 2.1.2 Cell lines and culture conditions

BHK-21 (ATCC CCL-10), TS/A (Nanni et al., 1983), CT26 (ATCC CRL-2638) and HEp-2 cells (ATCC CCL-23) were cultured in IMDM (Gibco BRL, Eggenstein, Germany) supplemented with 10% heat inactivated FCS (Integro, Zaandam, The Netherlands) and 0.25 mM β-mercaptoethanol (Serva, Heidelberg, Germany). Cells were cultured at 37°C and 5% $CO_2$ in a humidified atmosphere.

### 2.1.3 Antibodies

DRAQ5 (Biostatus, Leicestershire, United Kingdom)

Donkey-anti-goat-cy3 (R&D Systems GmbH, Wiesbaden, Germany)

goat-anti-*E. coli* (Biomol, Hamburg, Germany),

goat-anti-luciferase (Promega, Madison, USA)

goat-anti-rabbit Alexa 488 (Sigma-Aldrich, Deisenhofen, Germany)

rabbit-anti-goat Alexa 488 (Invitrogen, Karlsruhe, Germany)

rabbit-anti-*S. flexneri* (Biomol, Hamburg, Germany),

rabbit-anti-*S. typhimurium* (Sifin, Berlin, Germany)

rat-anti-CD11b PE (eBioscience, San Diego, USA)

rat-anti-mouse Fc receptor (Pharmingen, San Diego, USA),

rat-anti-mouse Gr-1 biotinylated (Pharmingen, San Diego, USA),

Streptavidin-cy5 (Invitrogen, Karlsuhe, Germany),

Phalloidin Alexa Fluor 594 (Invitrogen, Karlsruhe, Germany)

* rat-anti-Gr1 (RB6-8C5)

* rat-anti-Gr1 biotin (RB6-8C5)

* rat-anti-Gr1 FITC (RB6-8C5)

* The three latter antibodies were isolated from culture supernatants and biotinylated or FITC-conjugated according to standard procedures.

All other materials will be described within the methods.

## 2.2 Methods

### 2.2.1 Molecular biological methods

#### 2.2.1.1 Bacterial strains and growth conditions

The *E. coli* strains TOP10 and DH5α were purchased from Invitrogen (Karlsruhe, Germany). Both bacterial strains were used for general cloning and were grown in Luria-Bertani medium (LB) supplemented with the appropriate antibiotics and with 15 g/l agar, where appropriate (Sambrook, 1989). Bacteria were grown at 37°C in liquid medium with vigorous shaking. For infection experiments, *E. coli* TOP 10 was grown in LB broth supplemented with 30 µg/ml streptomycin (Sigma, Taufkirchen, Germany) or on LB agar plates supplemented with 30 µg/ml streptomycin at 37°C.

*S. typhimurium* strain SL7207 (hisG, ΔaroA) was kindly provided by Dr. Stocker (Hoiseth and Stocker, 1981). It was grown in LB medium supplemented with 30 µg/ml streptomycin at 37°C with vigorous shaking or on LB agar plates with 30 µg/ml streptomycin at 37°C.

*S. flexneri* strains *S. flexneri* M90T (Serotyp 5, Δdap) and *S. flexneri* (Serotyp 2a, ΔaroD) were kindly provided by Dr. Sansonetti (Sansonetti et al., 1982). *Shigellae* were grown in tryptic soy broth (TSB) or on TSB-agar plates (Beckton Dickinson, Heidelberg, Germany) supplemented with 200 µmol/l Congo red (Serva, Heidelberg, Germany) at 37°C. Medium for *S. flexneri* Δdap was additionally supplemented with 30 µg/ml kanamycin, 100 µg/ml DAP (Fluca, Buchs, Switzerland). Ampicillin was added at 50 µg/ml where indicated.

### 2.2.1.2 Expression plasmids

#### pCMVβm2A

pCMVβm2A is a eukaryotic expression plasmid that was constructed by exchanging the pUC replicon of the high copy number plasmid pCMVβ (Clontech, Palo Alto, USA) with the low copy replicon pMB1. It is a reporter plasmid with the gene encoding β-galactosidase under the control of the cytomegalovirus immediate early promoter (CMV IE). Its construction was described in Bauer, 2004 and Bauer et al., 2005.

#### pCMVlucm2A

The eukaryotic expression plasmid pCMVlucm2A is a reporter plasmid for the expression of firefly luciferase in mammalian cells driven by the CMV IE promoter. Besides the promoter and the firefly luciferase gene, it contains a splice donor/splice acceptor site (SV40 SD/SA), a polyadenylation signal from simian virus 40 (SV40 late polyA), the low copy number pMB1 replicon from pBR322 (pMB1ori) (Sutcliffe, 1979) and an ampicillin resistance gene (AmpR). Its construction is described in Zelmer, 2005.

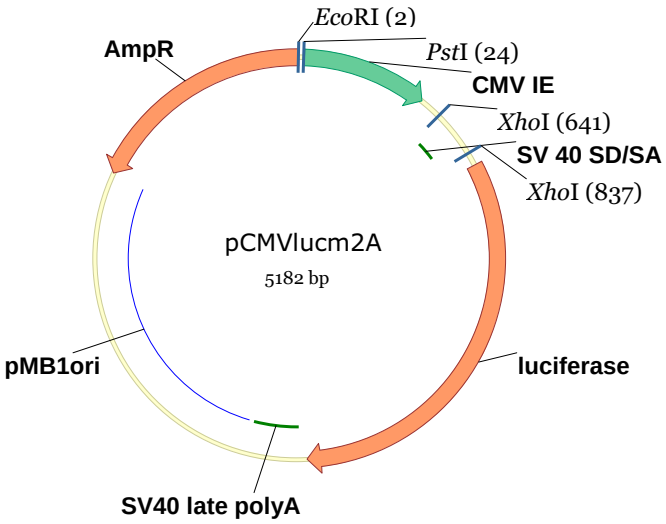



Fig. 2.1: Plasmid map of pCMVlucm2A. This reporter plasmid mediates the expression of the firefly luciferase gene via the eukaryotic CMV promoter.

#### pCMVluc-λ2In-m2A

Since the CMV promoter can be used by several Gram-negative bacteria (Goussard et al., 2003), an intron was introduced into the firefly luciferase of pCMVlucm2A, to be able to distinguish eukaryotic from bacterial gene expression. Therefore, the intron from the VJ gene

segment of the λ2 immunoglobulin gene of the mouse (Wu et al., 1982) was amplified and introduced via fusion PCR into the firefly luciferase gene of plasmid pCMVlucm2A. The exact mechanism and procedure of the fusion PCR is explained in chapter 2.2.3.1. The PCR product was then ligated into pCMVlucm2A via restriction sites SfuI/BsrGI.

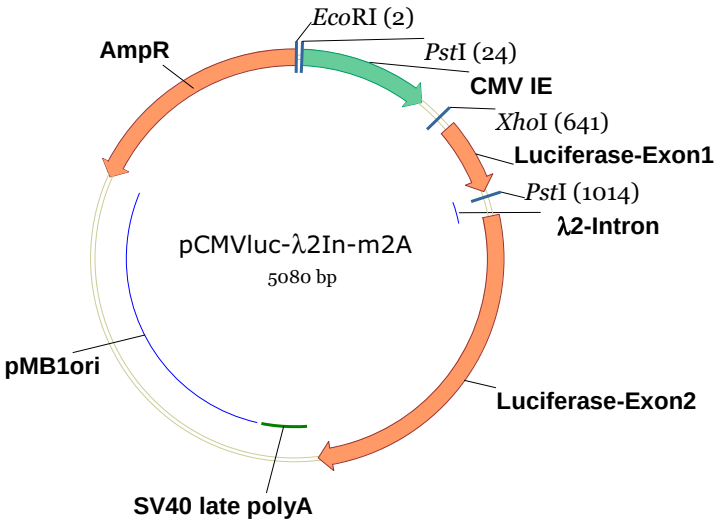



Fig. 2.2: Plasmid map of pCMVluc-λ2In-m2A. In this plasmid the firefly luciferase gene is interrupted by an intron of the λ2 immunoglobulin gene of the mouse to ensure the expression of luciferase exclusively in eukaryotic cells.

### 2.2.1.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction allows the selective amplification of DNA fragments between two regions of known sequence. Two oligonucleotides are used as primers, which bind to the complementary strands in opposite directions and, using a heat-stabile polymerase, the flanked DNA sequence can be amplified exponentially by repetitive cycles of denaturation and elongation.

Reaction mix:
1.0 µl primer 1 (10 pmol/µl)
1.0 µl primer 2 (10 pmol/µl)
1.0 µl template DNA
2.0 µl 10x PCR buffer
0.4 µl nucleotide mix (10mM each nucleotide)
0.1 µl AmpliTaq Gold™ (5U/µl)
14.4 µl $H_2O$

The reaction was conducted in 200µl reaction tubes (Applied Biosystems, Hamburg, Germany) in a primus 96 thermocycler from MWG-biotech (Ebersberg, Germany).

**Standard PCR conditions:**

After a 10 min activation of the AmpliTaq Gold™ polymerase (Applied Biosystems, Darmstadt, Germany) at 37°C, 30 cycles of amplification with a denaturation temperature of 94°C (30 sec), an annealing temperature of 58°C (30 sec) and an elongation temperature of 72°C (30 sec) were performed. The PCR was finished with a final extension at 72°C for 5 min.

**Fusion PCR**

To introduce a functional intron into the luciferase reporter gene, three requirements had to be fulfilled: 1. the intron had to be inserted at the beginning of the luciferase gene to ensure the expression of a nonsense protein in bacteria, which are unable to splice the two exons. 2. The number of nucleotides in the intron had to be not divisible by three, to ensure a frameshift and thus the expression of a nonsense protein, if the gene is not spliced correctly. 3. The intron had to contain the splice donor/splice acceptor site, which additionally should be flanked by the last two nucleotides of the preceding exon and the first two nucleotides of the succeeding exon.

As the original λ2-intron is flanked by the nucleotides AG and GA, respectively, the cDNA of the luciferase gene in pCMVlucm2A was screened for an AGGA-sequence that is framed by two single cutters for easier handling of the fragments. After the discovery of an AGGA-sequence that was framed by the two single cutters SfuI and BsrGI, respectively, the following four primers were constructed:

Lambda2In1-for: 5’-GAGTACTTCGAAATGTCCGTT-3’;
Lambda2In1-rev: 5’-GCAAATCGAGAGACAAGATGTGTATTGTTGCATACCCACTGC
AGTGTAGAAAGGCTGCTGACCGATAAATAACGCGCCCAA-3’;
Lambda2In2-for: 5’-CAATACACATCTTGTCTCTCGATTTGCTACTGATGACTGGATT
TCTTACCTGTTTGCAGGAGTTGCAGTTGCGCCCGCG-3’;
Lambda2In2-rev: 5’-CGAACGTGTACATCGACTGAA-3’;

The first primer binds to the luciferase gene and includes the restriction site for SfuI. The second primer is a reverse primer that includes the first two-thirds of the sequence of the λ2-intron. The third primer is a forward primer and includes the last two-thirds of the λ2-intron-sequence. The last primer is a reverse primer that binds to the luciferase gene and includes the

BsrGI restriction site. As the original λ2-intron consists of 93 bp and is thus divisible by three, one extra nucleotide was inserted into the sequence, which is marked in red. Two standard PCRs were performed with plasmid DNA from pCMVlucm2A as a template and with primers 1 and 2 for the first PCR, with primers 3 and 4 for the second PCR. After a gel-purification of the amplified PCR-fragments on a 2% (w/v) agarose gel, a third PCR, the fusion PCR, was performed. This PCR was accomplished with primers 1 and 4 and with the two gel-purified PCR-products as templates. The thermocycler conditions were always standard PCR conditions. Fig. 2.3 shows a schematic overview of the fusion PCR.

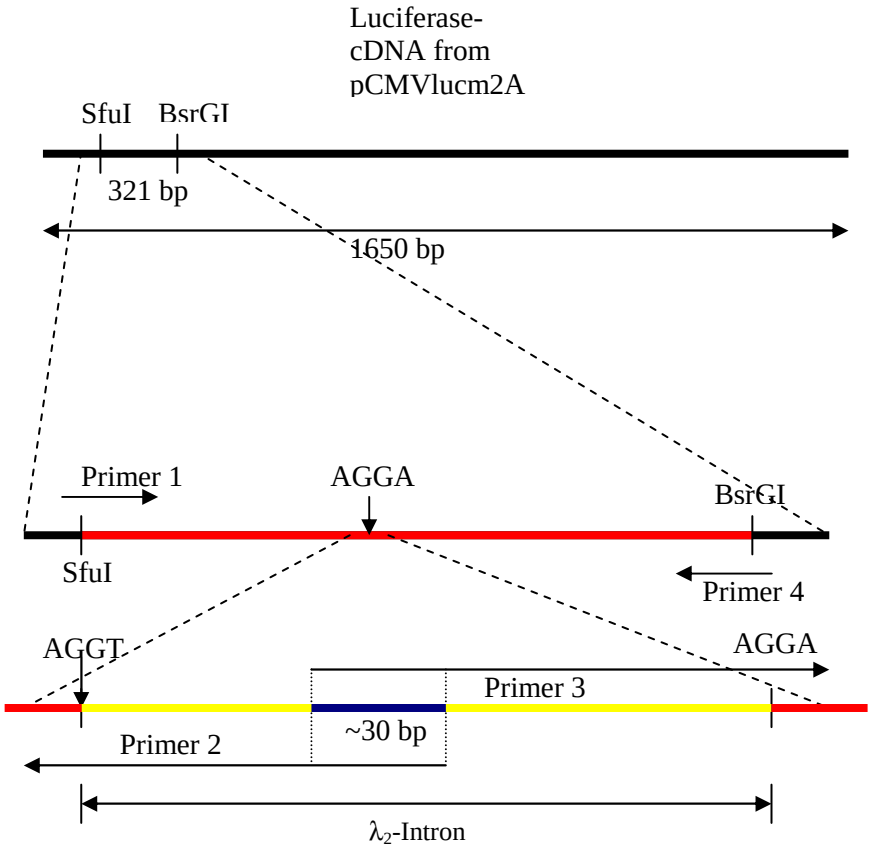



Fig. 2.3: Schematic overview of the fusion PCR. The exact mechanism is described in the text.

### 2.2.1.4 Agarose gel electrophoresis

DNA is negatively charged in aqueous solutions due to the ionization of its phosphate residues. Thus, linear, double-stranded DNA-fragments can be separated in an electric field according to the molecular size of the fragments. In this study agarose gels (SeaKem® LE Agarose, Cambrex) were used at concentrations of 0.8% and 2% (w/v), respectively. The gels were running in 1xTAE buffer at 110-120 V. DNA fragments were stained with ethidium bromide and visualized using the Herolab GmbH software.

| | |
|---|---|
| 50x TAE: | 242 g Tris |
| | 57.1 ml acetic acid |
| | 100 ml 0.5 M EDTA (pH 8.0) |
| | Filled up with $H_2O$ to 1l and autoclaved |
| DNA marker: | SmartLadder SF, 100bp-1kb (Eurogentec) |
| | mixture of HindIII-digested l-DNA and HaeIII-digested pFX174-DNA (MBI-Fermentas, St. Leon-Rot, Germany) |

Electrophoresis was conducted using the Horizon 11.14 system (Gibco BRL, Eggenstein, Germany)

**Isolation of DNA from agarose gels**

The extraction of DNA fragments from agarose gels for the later ligation was performed with the "QIAquick Gel Extraction Kit" from QIAGEN according to the manufacturer's protocol. DNA was always eluted in 50 µl water and stored at –20°C until use.

### 2.2.1.5 Molecular cloning

Molecular cloning comprises the cleavage of plasmid DNA with restriction enzymes and joining to foreign DNA fragments by the use of ligase. The resulting recombinant plasmids are then used to transform bacteria.

**Cleavage of DNA with restriction enzymes**

Restriction endonucleases recognize short specific DNA sequences, generally ranging between 4 and 8 nucleotides, and cleave the DNA in this region. Digestion of DNA was carried out using optimal buffer and temperature conditions for each enzyme according to the manufacturer's protocol. Finally, an aliquot of the digestion mix was analyzed by agarose gel electrophoresis for complete digestion.

**Ligation of DNA**

Ligation of a foreign DNA fragment to a linearized plasmid vector involves the formation of phosphodiester bonds. T4 DNA ligase (Gibco BRL, Eggenstein, Germany) was used to catalyze the formation of a phosphodiester bond between juxtaposed 5'phosphate and 3'hydroxyl termini in doublestranded DNA. Re-circularization of vector DNA can be limited by performing the ligation reaction with an excess of foreign DNA and a comparatively low concentration of the plasmid vector. Thus, vector and fragment, which were previously

digested with the same or compatible restriction enzymes, were ligated in a molar ratio of 1:10 and incubated at room temperature for 60 minutes.

#### 2.2.1.6 Transformation of bacteria

Different methods of transformation were carried out to introduce plasmid DNA into bacteria.

**Transformation of chemically competent *E. coli***

Chemically competent *E. coli* (Invitrogen, Karlsruhe, Germany) were thawed on ice, mixed with 10 µl of the ligation reaction or with 1 µl of plasmid DNA by gently tapping and were incubated for 30 minutes on ice. After a subsequent incubation for 30 seconds at 42°C, the bacteria were immediately placed back on ice for 5 min. 250 µl of SOC medium (supplied with the bacteria) were added to the bacteria and the suspension was incubated at 37°C and 180 rpm for one hour. Afterwards the bacteria were plated on LB plates containing the appropriate antibiotics and the plates were incubated at 37°C over night. The next day, single colonies were picked and cultivated in LB medium containing the appropriate antibiotics. The liquid cultures were incubated at 180 rpm and 37°C for 6 to 8 hours. Afterwards the clones were analyzed by plasmid isolation and restriction digestion.

**Transformation of electrocompetent *S. flexneri***

The electrocompetent bacteria were thawed on ice, mixed with 2 µl of the desired plasmid solution by gently tapping and incubated for 1 minute on ice. The suspension was transferred into a cuvette and electroporation was carried out (parameters 2.5 kV, 25 µF, 200Ω) with a Gene Pulser (Biorad). Immediately, 900 µl of TSB medium (+ DAP for *S. flexneri* Δdap) were added to the cuvette. The suspension was transferred to a tube and was incubated at 180 rpm at 37°C for one hour before plating on TSB plates containing the appropriate antibiotics. The plates were incubated at 37°C over night. Picking of colonies was performed as described above.

#### 2.2.1.7 Isolation of plasmid DNA

Depending on the required amount of plasmid DNA, mini or maxi plasmid preparations were performed to isolate plasmids from bacteria. To purify plasmid DNA from *E. coli* strains, the “GFX Micro Plasmid Prep Kit” (Amersham Biosciences) was used for analytical and the "Qiagen Plasmid Maxi Kit“ (Qiagen, Hilden, Germany) for preparative purposes. The “Qiagen Plasmid Mini Kit” (Qiagen, Hilden, Germany) was employed for purification of plasmid DNA from *Shigella* strains.

#### 2.2.1.8 Optical determination of DNA concentration

For optical determination of the DNA concentration, the extinction of a sample was measured at 260 nm. The $OD_{260}$ of 1 corresponds (at a diameter of the cuvette of 1 cm) to a DNA concentration of 50 µg/ml.

Photometer: BioPhotometer (Eppendorf, Hamburg, Germany)

#### 2.2.1.9 Long-term storage of bacteria

For the long-term storage of bacteria 700 µl of a fresh bacterial culture were mixed with 300 µl of 50% glycerin and stored at –80°C.

50% glycerin: 50% (v/v) glycerin in $H_20$, filter sterilized and stored at 4°C.

### 2.2.2 Tissue culture

#### 2.2.2.1 Determination of viable cells

An aliquot of a cell suspension was diluted 1:1 with 0.5% (w/v) trypan blue in PBS and counted into a Neubauer chamber. 0.1mm/0.025mm$^2$ (Brand, Wertheim, Germany). Only viable cells, whose plasma membranes are, unlike dead cells, not leaky for trypan blue and are therefore not stained, were considered.

#### 2.2.2.2 Propagation of adherent cells

For transfer of adherent growing cells to new culture flasks, cells were washed once with PBS, removed enzymatically from the substrate by adding trypsin-EDTA solution (TE) and incubated at 37°C in a humified atmosphere until the cells were detached. TE was inactivated by addition of FCS containing medium. The cells were harvested by centrifugation, washed once with PBS and resuspended in medium.

| | |
|---|---|
| PBS: | 137 mM NaCl; 2.6 mM KCl; 6.4 mM $Na_2HPO4$ x 2 $H_2O$; 1.4 mM KH2PO4; in $H_2O$ (pH 7) |
| TE: | 0.5 g/ml trypsin; 0.2 g/ml EDTA; in PBS |

#### 2.2.2.3 Cryoconservation of mammalian cells

Cells from a dense growing 12- or 6-well plate were taken in 0.5 ml ice-cold freezing medium. The cell suspension was temporarily frozen at -70°C and finally stored in liquid nitrogen.

Freezing medium: 10 % (v/v) dimethylsulfoxide (Merck, Darmstadt, Germany) in FCS

#### 2.2.2.4 Thawing of cells

The frozen cells were thawed at 37°C in a water bath and the cell suspension was taken up in medium. Afterwards, the cells were harvested by centrifugation, washed once and resuspended in medium.

### 2.2.3 Calcium phosphate transfection

Transfection represents a method for the transfer of DNA into cell culture cells. The principle of calcium phosphate precipitation is based on the formation of an insoluble precipitate of calcium phosphate, to which the DNA can be adsorbed. These calcium phosphate-DNA-complexes are taken up by the cells via endocytosis. The DNA reaches the nucleus, where it can be transcribed (transient transfection). Some of the DNA can be integrated into the genome of the recipient cell (stable transfection), which can subsequently transmit this DNA segment to the next cell generation. Practically: $3x10^5$ adherent cells per 25 $cm^2$ culture flask were seeded the day before. The medium was exchanged for 5 ml of fresh medium 4 h before transfection. For preparation of the calcium phosphate/DNA coprecipitate, 250 µl of the $CaCl_2$- DNA-mixture were added drop by drop under gentle mixing to 250 µl HEBS-buffer (2x) and incubated for 30-60 min at room temperature. The precipitate was then added to the medium above the cells and the transfected cells were cultured at 37°C. Excess of precipitate was removed the next day by exchange of medium. 24 h after exchange of the medium, transgene expression was analyzed (chapter 2.2.6).

HEBS-buffer (2x): 280 mM NaCl; 50 mM HEPES; 1.5 mM $Na_2HPO_4$ x 2 $H_2O$; add 100 ml; adjust with 5 M NaOH to pH 7,1; sterile filtering, aliquots stored at -20°C

$CaCl_2$-DNA-mixture: 25 µl $CaCl_2$ (250 mM); 10 µg co-transfer-DNA; $H_2O$ add 250 µl

### 2.2.4 *S. flexneri*-mediated transfer of eukaryotic expression plasmids to mammalian cells

Cells were seeded 24 h before infection in 24-well plates at a density of $5x10^4$ cells/well, resulting in approximately $1x10^5$ cells/well at the time of infection. Overnight cultures of *S. flexneri* strains were diluted 1:50 in the corresponding medium and grown at 37°C and 180 rpm for 2.5 hours. After washing once with PBS, bacteria were added to cell cultures at the indicated multiplicity of infection (MOI) and in a total volume of 200 µl medium. For *S. flexneri* Δdap, PBS and culture medium were supplemented with 100 µg/ml DAP. Subsequently, infection cultures were centrifuged to enhance invasion and incubated for 1.5-2 hrs at 37°C. Cells were then washed twice with PBS and cultured in medium supplemented with 50 µg/ml gentamycin to kill extracellular bacteria. Cells were incubated for the indicated time periods at 37°C and transient transgene expression was analyzed (chapter 2.2.6).

### 2.2.5 Infection of tumor-bearing BALB/c mice with *S. flexneri*, *S. typhimurium* and *E. coli*

6 week old, female BALB/c mice (Harlan, Borchen, Germany) were subcutaneously inoculated at the abdomen with $5x10^5$ cells of the colon adenocarcinoma cell line CT26 or with $1x10^6$ cells of the adenocarcinoma cell line TS/A. Mice bearing tumors of approximately 5-7 mm diameter (day 10 post infection) were intravenously injected with $5x10^6$ CFU of *S. typhimurium* or *E. coli* suspended in phosphate-buffered saline (PBS) and intratumorally with $1x10^7$ *S. flexneri* Δdap suspended in PBS with 100 µg/ml DAP, respectively. At the indicated time points mice were sacrificed and their tumors and spleens were transferred into 1 ml, livers were transferred into 2 ml of sterile ice-cold PBS containing 0,1% (v/v) Triton X-100. Tissues were homogenized by using a Polytron PT3000 homogenizer (Kinematica).

For determination of total CFU per organ, bacteria homogenates were serially diluted in PBS, in case of *Shigella* they were serially diluted in PBS with 100 µg/ml DAP, plated with the required antibiotics and colonies were counted.

For transgene analysis, the tissue homogenates were analyzed as described in chapter 2.2.6.

### 2.2.6 Quantitation of luciferase expression

To detect luciferase, the Dual Glo luciferase assay system (Promega, Mannheim, Germany) was used according to the manufacturer's recommendations. 10 µl of each sample were

measured and luminescence was recorded for 10 s using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

## 2.2.7 Immunological methods

### 2.2.7.1 Neutrophil depletion

For depletion of neutrophils, mice received three doses of 25 µg monoclonal rat-anti-Gr1 (RB6-8C5) antibody diluted in 100 µl PBS intraperitoneally (i.p.) 1 day before, simultaneously and 1 day after infection. Depletion was controlled by taking blood samples from the treated mice and analyzing the blood via flow cytometry.

### 2.2.7.2 Flow cytometry of blood samples

Erythrocytes of 50 µl blood were lysed in 1.5 ml erythrocyte lysis buffer (ELB), vortexed, incubated for 5 min at room temperature (RT) and centrifuged for 5 min at RT. This procedure was repeated once in 1 ml ELB. Cell pellets were then washed once with PBS and stained with rat-anti-Gr1 FITC and rat-anti-CD11b PE for 20 min on ice. Afterwards, cells were washed once with PBS and analyzed on the FACSCalibur (Beckton Dickinson, Heidelberg, Germany). Data were then analyzed with CellQuestPro software (Becton Dickinson, Heidelberg, Germany).

ELB: 2.06 g Tris (HCl); 7.49 g $NH_4Cl$; filled up to 1l with $H_2O$ (pH 7.2)

### 2.2.7.3 Histology

Tumors were removed from sacrificed mice and snapfrozen in Tissue-Tek OCT Compound (Sakura Finetek, Zoeterwoude, The Netherlands). Cryosections of 10 µm were cut with a microtome-cryostat (Cryo-Star HM560V, Microm, San Marcos, USA) and placed onto slides. Slides were air dried at room temperature overnight and fixed in acetone at -20°C for 3 min. Slides were rehydrated in PBS, blocked with 50 µg/ml BSA and 1 µg/ml FcR blocker (rat-anti-mouse CD16/CD32), and stained with the following reagents: polyclonal rabbit-anti-*S. typhimurium*, polyclonal goat-anti-rabbit Alexa 488, polyclonal rabbit-anti-*S. flexneri*, polyclonal goat-anti-*E. coli*, polyclonal rabbit-anti-goat Alexa 488, rat-anti-Gr1 biotin, Streptavidin-cy5, rat-anti-CD11b PE, goat-anti-luciferase, donkey-anti-goat-cy3, Phalloidin Alexa Fluor 594 and DRAQ5. After staining, the slides were washed and dried, mounted with mounting medium (Neomount, Merck, Darmstadt, Germany) and analyzed using a laser scanning confocal microscope (LSM 510 META, Zeiss). Images were processed with LSM5 Image Browser (Zeiss) and Adobe Photoshop 7.0.

For paraffin sections, tumors were fixed in 10 % (v/v) paraformaldehyde and embedded in paraffin wax. 5 µm sections were mounted on starfrost slides and stained with hematoxilin and eosin. The stained paraffin sections were analyzed with an Olympus BX51 microscope and pictures were taken with an Olympus U-CMAD3 camera.

PBS/BSA buffer: 0.05% BSA in PBS, pH 7.2

### 2.2.8 Electron microscopy

#### 2.2.8.1 Transmission electron microscopy

Tumors were fixed in 5% formaldehyde and 2% glutaraldehyde, cut into cubes with an edge length of 3-5 mm and contrasted in 1% aqueous osmium tetroxide. Subsequently, the samples were dehydrated with a graded series of acetone (10, 30, 50, 70, 90, 100%) on ice for 30 min per step. The dehydrated samples were infiltrated with an epoxy-embedding resin (described by Spurr, 1969) (1 part acetone/1 part resin; 1 part acetone/2 parts resin, pure resin alternating). The infiltrated samples were polymerized at 70°C for 10 h and cut into ultrathin sections with a diamond knife. The sections were picked up with Formvar-grids and contrasted with uranylacetate and lead-citrate. Finally the samples were analyzed with a transmission electron microscope (TEM910 Zeiss, Germany) at an acceleration voltage of 80 kV.

#### 2.2.8.2 Scanning electron microscopy

Tumors were fixed in 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer. Afterwards they were washed three times in cacodylate buffer and dehydrated with a graded series of acetone (10, 30, 50, 70, 90, 100%) on ice for 30 min per step. After a double wash with 100% acetone at RT for 30 min a critical-point drying with liquid $CO_2$ was performed. The dried samples were fixed on sample brackets. A second sample bracket was applied and pressed slightly against the sample. By uncompressing the brackets, the samples were broken. The fractured surface was sputtered with a thin gold layer. Finally, the samples were analyzed with a Zeiss field emission scanning electron microscope DSM 982 Gemini at an acceleration voltage of 5 kV. Images were digitally stored on MO-disks.

Cacodylate buffer: 0.1 M cacodylate; 0.01 M $CaCl_2$; 0.01 M $MgCl_2$; 0.09 M saccharose; pH 6.9

# 3 Results

The transfer of eukaryotic expression plasmids from intracellular invasive bacteria into mammalian host cells *in vitro* and *in vivo* has been reported repeatedly (Sizemore et al., 1995; Courvalin et al., 1995; Darji et al., 1997; Hense et al., 2001). Regarding tumor therapy, most *in vivo* approaches that exploit bacteria-mediated gene-transfer generally aim at the induction of immune responses against tumor antigens and at the development of cancer vaccines. Even though bacterial gene transfer has often been proposed as a means to deliver therapeutic molecules – or more exact, to deliver plasmids that encode therapeutic molecules – into solid tumors, no such system has been described yet.

In the present work two attenuated *S. flexneri* strains were explored to deliver a plasmid encoding luciferase as a reporter gene into different cancer cell lines *in vitro* and into an established solid carcinoma *in vivo*. Although the *in vitro* results obtained from infection studies with different cancer cell lines were very promising, subsequent *in vivo* experiments gave fluctuating results.

The *in vivo* delivery of plasmids into mammalian tumor cells appears to be more complex and includes unknown variables such as efficient tumor colonization by bacteria, impact of tumor microenvironment on bacteria, interactions of bacteria and of tumor cells with the immune system, etc. Hence, it became clear that for the establishment of an efficient bacteria-mediated plasmid delivery system, all such variables had to be considered and their possible influence on bacterial gene transfer needed to be investigated. Therefore, the intratumoral settlement and the physiological state of *S. flexneri*, but also of *S. typhimurium* and *E. coli*, the concomitant intratumoral changes like infiltration of primary immune cells and their impact on bacterial colonization bacteria were studied in the present work.

## 3.1 *Shigella flexneri*-mediated gene transfer into solid tumors

Attenuated *S. flexneri* strains were amongst the first intracellular invasive bacteria to be shown to transfer eukaryotic expression plasmids into mammalian cells (Hone et al., 1988; Sizemore et al., 1995; Sizemore et al., 1997). The typical eukaryotic expression plasmid, which was also used by Sizemore et al. amongst others, encodes the gene of interest, which could be a therapeutic gene or a reporter gene, under the control of the immediate early promoter and enhancer of the human cytomegalovirus (CMV promoter). The CMV promoter has been proven to be a strong, constitutive promoter that allows high-level expression of

recombinant genes in a wide range of mammalian cells. In 2003 Goussard et al. found that the CMV promoter can also direct protein synthesis in Gram-negative bacteria such as *Shigellae*, *Salmonellae* and *E. coli*. Thus, whenever studying gene transfer from Gram-negative bacteria to mammalian cells using genes that are under the control of the CMV promoter, the results have to be interpreted with caution, unless eukaryotic expression is unequivocally demonstrated (Darji et al., 1997; Hense et al., 2001).

### 3.1.1 The CMV promoter directs protein synthesis in *S. flexneri* M90T Serotyp 5, Δdap and *S. flexneri* Serotyp 2a, ΔaroD

In order to test whether the two employed *S. flexneri* strains, *S. flexneri* M90T Serotyp 5, Δdap (*S. flexneri* Δdap) and *S. flexneri* Serotyp 2a, ΔaroD (*S. flexneri* ΔaroD), were able to use the CMV promoter for protein synthesis, both strains were transformed with the pCMVlucm2A plasmid (Fig. 2.1). In this eukaryotic expression plasmid the luciferase reporter gene is under the control of the CMV promoter. After overnight growth in TSB medium with the appropriate supplements at 37°C and aeration at 180 rpm, the overnight cultures were serially diluted, the bacteria were lysed and bacterial luciferase expression was measured. In concordance with the results from Goussard et al. (2003), both *S. flexneri* strains did use the CMV promoter to direct the transcription of the reporter gene luciferase. As can be seen in Fig. 3.1, the basal reporter gene expression in *S. flexneri* ΔaroD was higher than for *S. flexneri* Δdap for reasons unknown. In both strains, especially in *S. flexneri* ΔaroD, the bacterial luciferase expression might be high enough to be mistaken for successful bacteria-mediated gene transfer and expression in host cells.

### 3.1.2 Distinction between eukaryotic and bacterial gene expression

To overcome this problem and to be able to ultimately distinguish bacterial from eukaryotic gene expression, an intron was cloned into the luciferase reporter gene. In contrast to bacterial genes, the genes of eukaryotic cells are often interrupted by introns, which are non-coding sections of DNA. In the course of eukaryotic transcription, the whole eukaryotic genes, including the non-coding introns and the exons, i.e. the coding sections, are transcribed into pre-mRNA. From these primary transcripts the introns are spliced out to generate the mature mRNA. This mRNA is then translated into polypeptides. As the genes of prokaryotes are not interrupted by introns, they do not need to build spliceosomes, the ribo-protein complexes that perform the splicing process. Being unable to build spliceosomes, prokaryotes cannot splice introns.

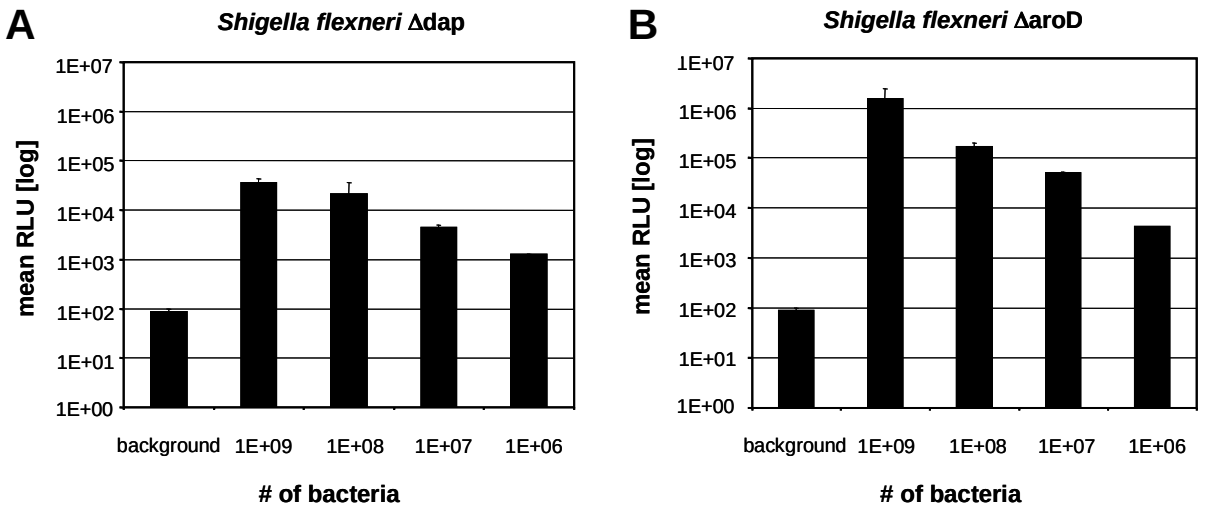



Fig. 3.1: The CMV promoter can direct protein synthesis in *S. flexneri* M90T Serotyp 5, Δdap and *S. flexneri* Serotyp 2a, ΔaroD. Both bacterial strains were transferred with the eukaryotic expression plasmid pCMVlucm2A. Luciferase expression was measured in serially diluted bacterial fractions after overnight growth. (**A**) Basal luciferase expression in *S. flexneri* Δdap. (**B**) Basal luciferase expression in *S. flexneri* ΔaroD. The graphs show mean values of at least three independent experiments.

Thus, by cloning a functional intron into the luciferase reporter gene, only eukaryotic cells should be able to express functional luciferase, while the expression by bacteria should result in a nonsense protein, which does not give a positive luciferase signal. To ensure correct splicing in mammalian cells and the expression of a nonsense protein in bacteria, the intron had to fulfill several requirements. It should be short because short fragments are easier to handle in fusion PCRs during the cloning procedure (compare p.50). Further, it should contain a number of nucleotides, which is not divisible by three, and thus result in a frameshift mutation and a nonsense protein in bacteria upon translation. For the same reason it should be inserted at the upstream region of the luciferase gene. Finally it should contain the splice donor/splice acceptor site, which additionally should be flanked by the last two nucleotides of the preceding exon and the first two nucleotides of the succeeding exon. The intron was selected from the VJ gene segment of the λ2 immunoglobulin gene of the mouse (Wu et al., 1982). As this 93 bp-intron is divisible by three, one extra nucleotide was inserted into the intron. The modified intron was cloned into pCMVlucm2A and resulted in the new plasmid pCMVluc-λ2In-m2A (Fig. 2.2).

First, the functionality of this plasmid had to be tested, i.e. whether mammalian cells were able to splice the λ2-intron and express functional luciferase, while no luciferase should be detected in the two *S. flexneri* strains. Therefore, mammalian BHK-21 cells were transfected with pCMVluc-λ2In-m2A via calcium phosphate precipitation including pCMVβm2A and pCMVlucm2A as negative and positive controls, respectively. 48 h after transfection, the cells

were harvested, lysed and 10 µl of undiluted lysate were tested for enzymatic activity of luciferase. Simultaneously, 1 ml of either of the overnight cultures of the two *S. flexneri* strains that were transformed with pCMVluc-λ2In-m2A was centrifuged, the bacteria were lysed and luciferase activity was determined. Fig. 3.2 shows that BHK-21 cells transfected with the intron-containing expression plasmid expressed higher activity of luciferase than BHK-21 cells transfected with the original plasmid. It is known that many genes are better expressed in eukaryotic cells when they contain an intron. This might also apply for luciferase. Transfection of BHK-21 cells with a control plasmid that encoded β-galactosidase instead of luciferase did not show any luciferase activity and marks background levels. No luciferase activity could be revealed for the two *S. flexneri* strains that were transformed with the newly constructed pCMVluc-λ2In-m2A plasmid. Both bacterial strains were not able to produce functional luciferase from this plasmid, although they could express luciferase from the initial expression plasmid (compare Fig. 3.1).

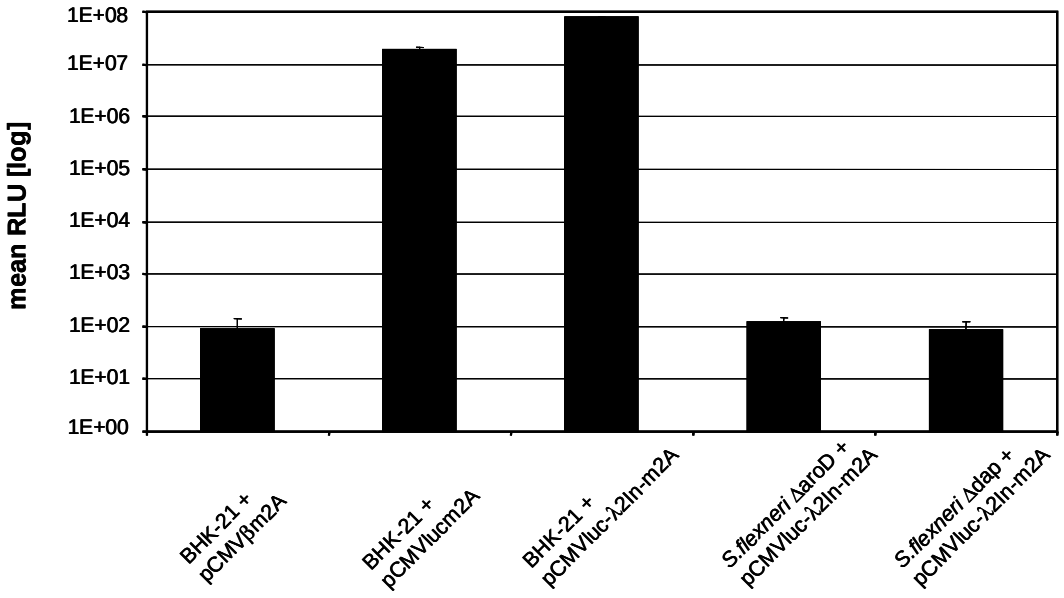



Fig. 3.2: Luciferase expression in BHK-21 cells 48 h after calcium phosphate transfection. BHK-21 cells were transfected with pCMVluc-λ2In-m2A, pCMVlucm2A and pCMVβm2A, respectively. The cells are able to express high amounts of functional luciferase from the λ2-intron containing reporter gene. Luciferase expression in *S. flexneri* strains that were transformed with pCMVluc-λ2In-m2A corresponds to background levels. The results show mean values of at least three independent experiments. Error bars show standard deviations.

Thus, pCMVluc-λ2In-m2A meets all the demands of a delivery vector for bacteria-mediated gene transfer into mammalian cells. It was therefore useful as a reporter-plasmid for bacterial

gene transfer experiments, as it allows the unequivocal distinction between mammalian and bacterial protein expression.

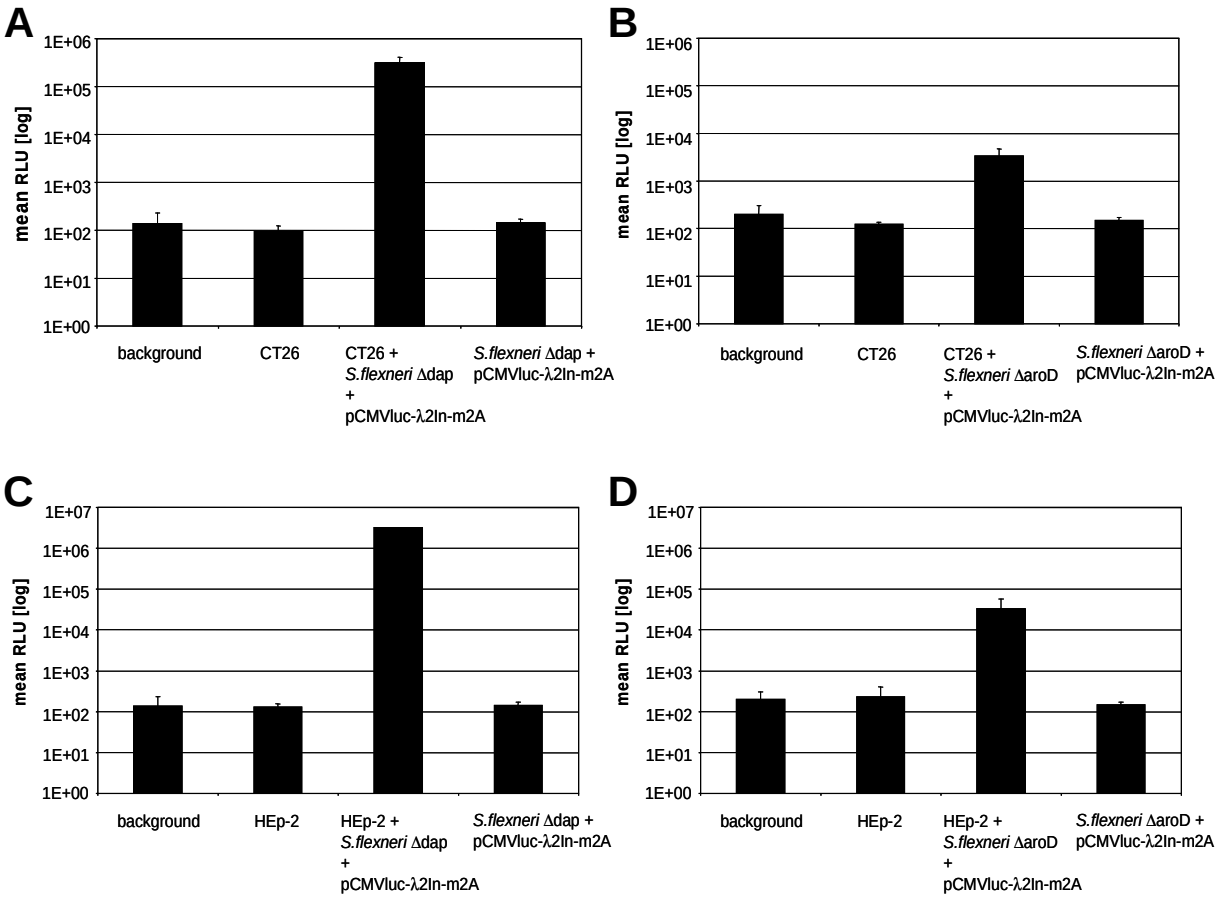



Fig. 3.3: *In vitro* bacteriofection of different tumor cell lines with attenuated *S. flexneri* strains carrying the expression plasmid pCMVluc-λ2In-m2A. Luciferase expression was determined 24 h after bacteriofection. Enzymatic activity of luciferase was determined in CT26 cells (**A**) and HEp-2 cells (**C**) that were bacteriofected with *S. flexneri* Δdap and in CT26 cells (**B**) and HEp-2 cells (**D**) that were bacteriofected with *S. flexneri* ΔaroD, respectively. Data show mean values of at least three independent experiments. Error bars show standard deviations.

### 3.1.3 *In vitro* gene transfer into different tumor-cell lines

After plasmid pCMVluc-λ2In-m2A had proven its functionality, it was used *in vitro* for bacterial gene transfer experiments into the mammalian tumor cell lines CT26 and HEp-2. To this end, tumor cells were infected with plasmid-bearing bacteria at an MOI of 250. Two hours after infection the culture medium was exchanged and Gentamycin was added to kill extracellular bacteria. 24 hours after infection, the cells were harvested, lysed and luciferase-expression was measured.

As expected, bacteria-mediated gene transfer into both tumor cell lines could be observed (Fig. 3.3). While uninfected tumor cells and both *Shigella* strains alone showed only background activity, both tumor cell lines did express luciferase 24 hours after infection with pCMVluc-λ2In-m2A-bearing *Shigellae*. HEp-2 cells (Fig 3.3 C and D) exhibited a luciferase activity that was 10-fold higher than CT26 cells (Fig. 3.3 A and B).

When comparing gene transfer efficiency between *S. flexneri* Δdap (Fig. 3.3 A and C) and *S. flexneri* ΔaroD (Fig. 3.3 B and D), the first strain transferred plasmids noticeably more efficient than the second strain, resulting in luciferase activities in the tumor cells that were 100-fold higher. This phenomenon might be due to the fact that *S. flexneri* Δdap is impaired in its cell wall synthesis and is not able to replicate without supplementary diaminopimelic acid (DAP) in the medium. As DAP is not produced by the tumor cells, the bacteria should lyse quickly inside the cells and release their plasmid load into the cytosol (Loessner et al., 2006). Probably, these bacteria release plasmids more efficiently into the cytoplasm than *S. flexneri* ΔaroD, whose cell wall synthesis is not impaired.

### 3.1.4 *In vivo* gene transfer into solid CT26 tumors

Having shown promising results *in vitro*, the *S. flexneri*-mediated plasmid transfer was tested *in vivo*. As *S. flexneri* Δdap was more efficient in gene transfer experiments *in vitro* than *S. flexneri* ΔaroD, all *in vivo* experiments were performed with *S. flexneri* Δdap.

Studies of Zelmer (2005) revealed that *S. flexneri* do not preferentially accumulate in solid tumors when given systemically. Thus, they had to be administered directly into the tumor. Accordingly, $5x10^6$ *S. flexneri* Δdap carrying the eukaryotic expression plasmid pCMVluc-λ2In-m2A were intratumorally injected into CT26 tumor-bearing BALB/c mice. Two days and three days after infection, mice were sacrificed, tumor, spleen and liver were removed, homogenized and luciferase activity in the tissue homogenates was measured. The results are summarized in Fig. 3.4. The experiment was repeated twice with similar results. The data shown represent mean values of luciferase activity of 3-4 mice per value.

Two days and three days after bacteriofection, eukaryotic transgene expression could be observed in CT26 tumors. Expression could also be detected in spleen and liver albeit to a lesser extend. The detection of luciferase activity in organs different from the tumor is not astonishing, as this *Shigella* strain had been shown to disseminate to spleen and liver after intratumoral administration and persists for at least 4 days post administration (Zelmer, 2005). As *Shigellae* are invasive and induce their own uptake into cells, they should be able to infect various cell types, i.e. cells of different organs, into which they could transfer their expression plasmids.

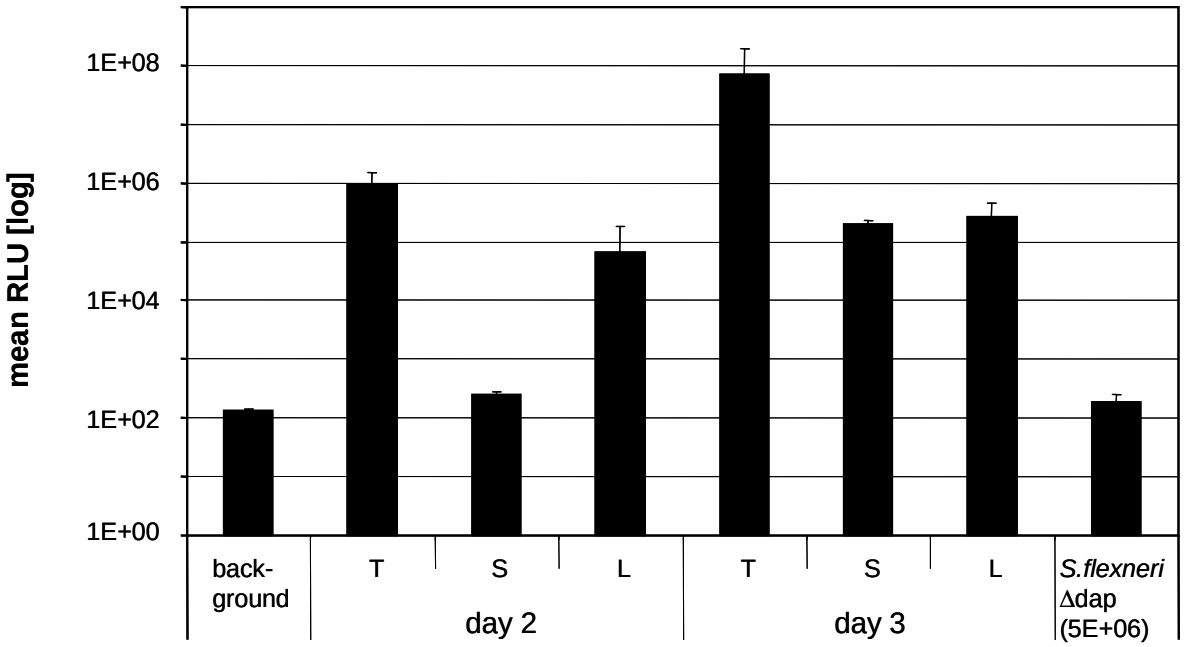



Fig. 3.4: *In vivo* bacteriofection of subcutaneous CT26 tumors (T), spleen (S) and liver (L) with attenuated *S. flexneri* Δdap carrying the expression plasmid pCMVluc-λ2In-m2A. Luciferase expression was determined 2 days and 3 days after bacteriofection. Data show mean values of 3-4 mice. Error bars show standard deviations. Experiments were repeated with identical results.

In general, the luciferase activity appears to be higher at later times post infection. Obviously, over time higher numbers of cells will become infected resulting in higher numbers of cells expressing the transgene after *Shigella*-mediated transfer of the expression plasmid. This showed that *in vivo* gene transfer into immunocompetent mice mediated by *S. flexneri* Δdap + pCMVluc-λ2In-m2A after intratumoral infection is generally possible.

However, after this initial series of functional *in vivo* gene transfer of pCMVluc-λ2In-m2A from *S. flexneri* Δdap into the cells of solid CT26 tumors, spleen and liver, fluctuating results were obtained. In the majority of cases *in vivo* gene transfer into the tumor cells was not successful, while spleen and liver sometimes displayed low positive luciferase activity. Intensive error analysis that aimed at improving gene transfer efficiency of the bacteria did not change the outcome of these experiments.

One characteristic of *S. flexneri* is its large virulence plasmid, which confers the invasive properties to these bacteria. As plasmids always represent a burden for the microorganism, *S. flexneri* tend to quickly lose this plasmid unless under appropriate selection pressure. One simple way to distinguish virulent from avirulent *Shigella* strains is their ability to bind Congo red. Avirulent clones, which have lost their virulence plasmid, fail to bind Congo red, while virulent clones do (Payne and Finkelstein, 1977). By strictly cultivating the bacteria in the

presence of Congo red, a clone containing up to 99% virulent bacteria could be obtained. These bacteria showed improved gene transfer *in vitro*, but could not influence the outcome of the *in vivo* results (data not shown).

*In vitro* systems represent highly artificial systems with a defined number of cell types – in this case only one – in which all the parameters can be controlled and optimized. In contrast, *in vivo* systems are extremely more complex with only limited options to optimize the different parameters. As the *in vitro* gene transfer constantly gave positive results, while *in vivo* gene transfer experiments transformed in parallel failed, the limiting steps had to be found in the mouse, especially in the interactions of tumor and immune cells with the bacteria.

Thus, when trying to establish a therapeutic method like bacteria-mediated gene therapy of solid tumors it is essential to know as many details of the system as possible. However, only few details are known of this experimental system. Bacterial distribution, influences of bacteria on the tumor and on the host's immune system as well as interactions of the bacteria with different host cells have not been described until now. Consequently, to understand the failure of bacteriofection of CT26 tumors *in vivo*, tumors of *S. flexneri*-infected and uninfected mice were analyzed and compared.

## 3.2 Distribution of *Shigella flexneri* Δdap in solid CT26 tumors

In order to determine where *Shigellae* are located inside solid tumors, CT26 tumor-bearing BALB/c mice were intratumorally infected with *S. flexneri* Δdap. Two days after infection tumors were removed, snapfrozen and cut into 10 µm sections. The cryosections were stained with Alexa 488-labeled antibodies that detect the bacteria, Phalloidin, which stains the cytoplasm and DRAQ5, which stains the DNA. Fig. 3.5A gives an overview of an intratumorally *S. flexneri* Δdap-infected CT26 tumor. The majority of bacteria accumulate inside a large area with loosely attached cells. This area was shown to be largely necrotic (compare Fig. 3.22). Bordering this area a layer of densely packed cells surrounding the necrosis was observed. Here, most of the *Shigellae* were located, as shown in Fig. 3.5 A. The *Shigellae* appear to form a ring that is associated with the densely packed cells. Fig. 3.5 B displays a higher magnification of this region.

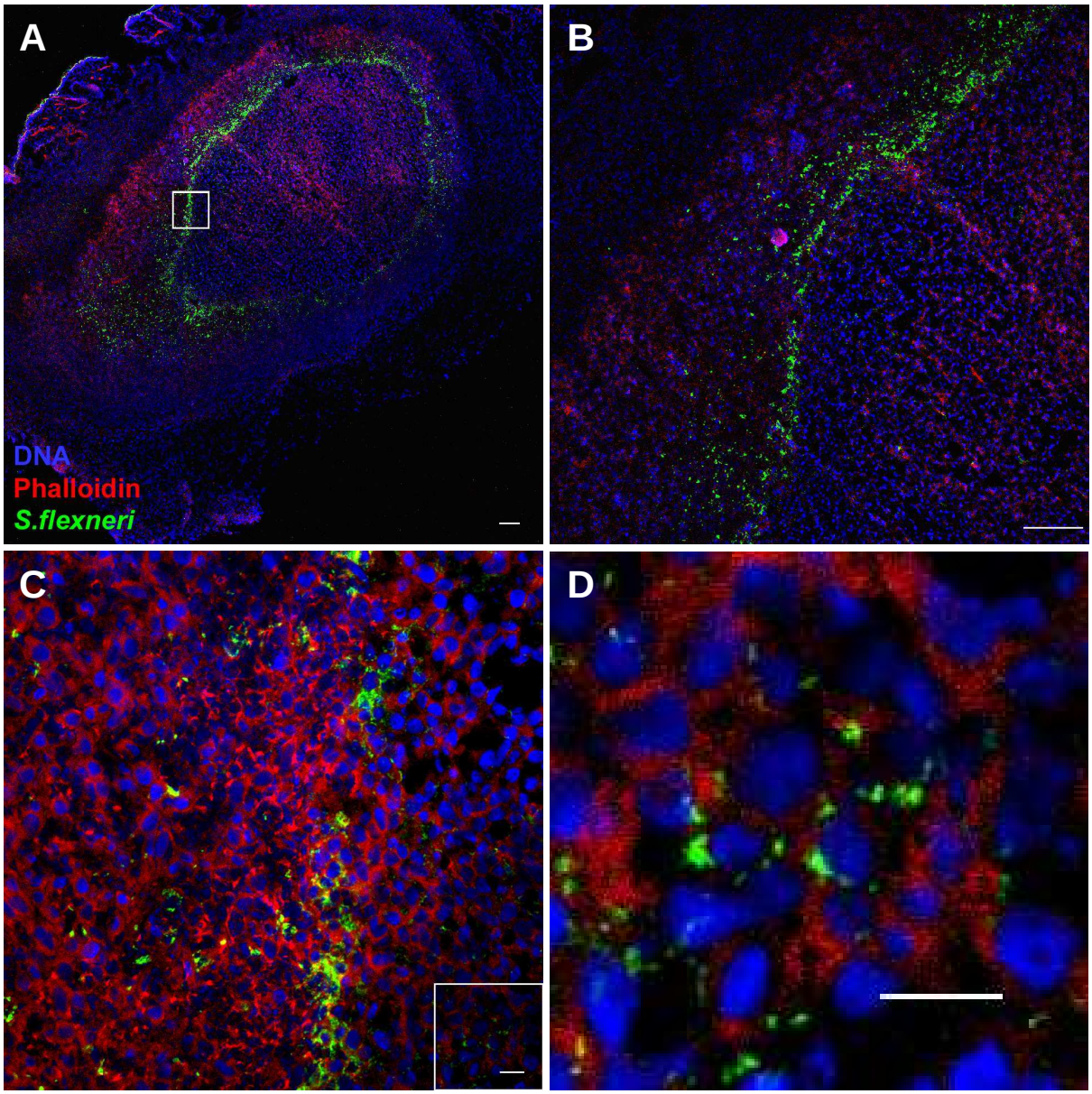



Fig. 3.5: Distribution of *S. flexneri* Δdap in solid CT26 tumors 2 days post intratumoral infection. Bacteria are stained in green, DNA is stained in blue and the cellular actin is stained in red. (**A**) Low magnification overview of an *S. flexneri* colonized tumor. (**B**, **C**) Higher magnifications of the border between viable and necrotic tumor tissue. (**D**) High magnification of the bacteria-colonized, necrotic region. The white box in **A** indicates the area of enlargement in **C**, while the white box in **C** indicates the area of enlargement in **D**. The white bars represent 100 µm (**A** and **B**) and 10 µm (**C** and **D**), respectively. The pictures are representative for at least 3 different *S. flexneri* Δdap infected CT26 tumors from independent experiments.

In Fig. 3.5 C the transition between the necrotic region (right) and the densely packed region (left) is more enlarged, demonstrating the accumulation of bacteria between the necrotic cells and the densely packed cells. Most of the necrotic cells still have an intact nucleus while only little of the cytoplasm is left. Fig. 3.5 C is a magnification of the white box indicated in Fig.

3.5 A. Fig. 3.5 D is a magnification of the white box in Fig. 3.5 C, which shows single bacteria in the necrotic tumor tissue.

Only very few bacteria could be detected in vital tumor tissue, distant from the necrotic area. However, some bacteria could also be found to accumulate in larges clusters directly below the skin spanning the tumor (Fig. 3.6).

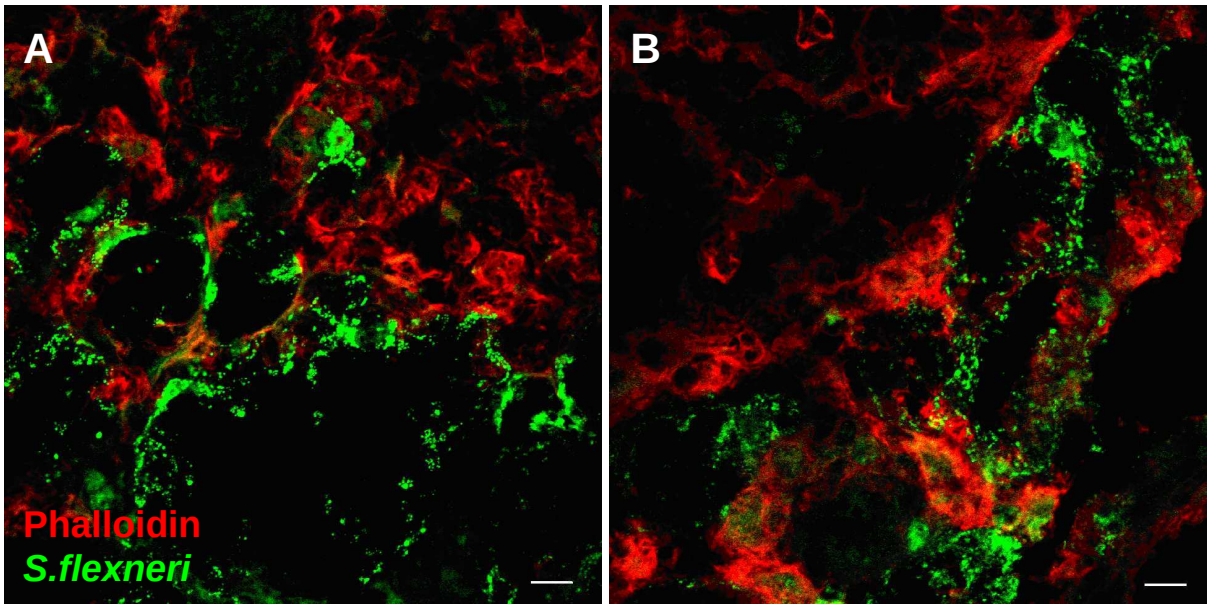



Fig. 3.6: Accumulation of *S. flexneri* Δdap directly below the skin of CT26 tumors. Bacteria are stained in green, cellular actin is stained in red. (**A**) and (**B**) show high magnification images of bacterial accumulation in two different CT26 tumors. The white bars represent 10 µm. The pictures are representative for at least three different *S. flexneri* Δdap infected CT26 tumors from independent experiments.

The accumulation of bacteria directly under the skin might not be astonishing and could be explained by the route of infection. Since the tissue of uninfected, solid CT26 tumors is rather dense, only very small volumes (10-20 µl) can be injected intratumorally. Despite of careful injection, small amounts of the inoculum will leak out of the tumor through the injection channel when the needle is removed and some of the bacterial suspension will remain in the space directly below the skin. As a result, a significant number of bacteria should accumulate directly below the skin.

The inhomogeneous distribution of *S. flexneri* Δdap inside the solid tumors was rather surprising. Being an invasive facultative anaerobic bacterium, *S. flexneri* Δdap was expected to spread all over the tumor. Hypoxic areas should be colonized but also oxygenated areas and *Shigella*'s ability to actively spread from cell to cell should facilitate the dissemination. In contrast, *Shigellae* were almost exclusively found inside the large necrotic area and directly below the skin. Occasionally, bacteria were found in vital tumor tissue, associated with living

cells. Such bacteria might be the initiators for gene transfer that was occasionally observed, as gene transfer can only occur, when the bacteria are infecting living cells. The reason for the observed inhomogeneous distribution remained unclear at this time. However, the question arose, whether this kind of distribution inside a solid tumor was characteristic for *S. flexneri*, or whether different bacteria, e.g. naturally tumor-targeting bacteria behave similarly.

## 3.3 Distribution of *Salmonella typhimurium* SL7207 and *Escherichia coli* TOP10 in solid CT26 tumors

To confirm the tumor-targeting ability of the two strains *S. typhimurium* SL7207 and *E. coli* TOP10, BALB/c mice bearing a CT26 tumor were intravenously infected with either of the two strains. Two days after infection, tumor, spleen and liver were homogenized and the CFU per tissue were determined. Both bacterial strains exhibited a strong preference for the solid tumor (Fig. 3.7 A and 3.7 B). While the number of *Salmonellae* in the tumor was 50-100 times higher than in other tissues (Fig. 3.7 A), the *E. coli* strain used showed an even stronger preference for the tumor. Here, 50-100,000 times more bacteria were found in the tumor in comparison to other organs (Fig. 3.7 B).

In order to determine, where the bacteria accumulate inside the CT26 tumor, the tumors were removed two days p.i., snapfrozen and cut into 10 µm sections. Fig. 3.8 shows the distribution of *S. typhimurium* SL7207 two days after i.v. infection. Fig. 3.8 A gives an overview of the infected tumor tissue, while Fig. 3.8 B shows an enlargement of necrotic tumor tissue. Here, the loose association between tumor cells inside the necrosis is clearly visible. Again, most nuclei appear to be intact, while the cytoplasm is largely disintegrated. Fig. 3.7 C is a magnification of the white box marked in Fig. 3.7 A and highlights the border between viable (V) and necrotic (N) tumor cells. Fig. 3.7 D is an enlargement of the white box in Fig. 3.7 C and shows single bacteria in the necrotic tumor tissue.

Interestingly, *S. typhimurium* were found only inside the necrotic tumor tissue or at the rim between necrotic and viable tumor cells and showed a similar distribution pattern as *S. flexneri*. However, *S. typhimurium* could not be detected under the skin covering the solid CT26 tumor. *S. typhimurium* entered the solid tumor via the tumor vasculature and were not directly administered into the tumor like *S. flexneri*. Thus, they have no direct access to the skin. The route of administration might also explain why *Salmonellae* were never found to be associated with viable cells distant from necrotic areas.

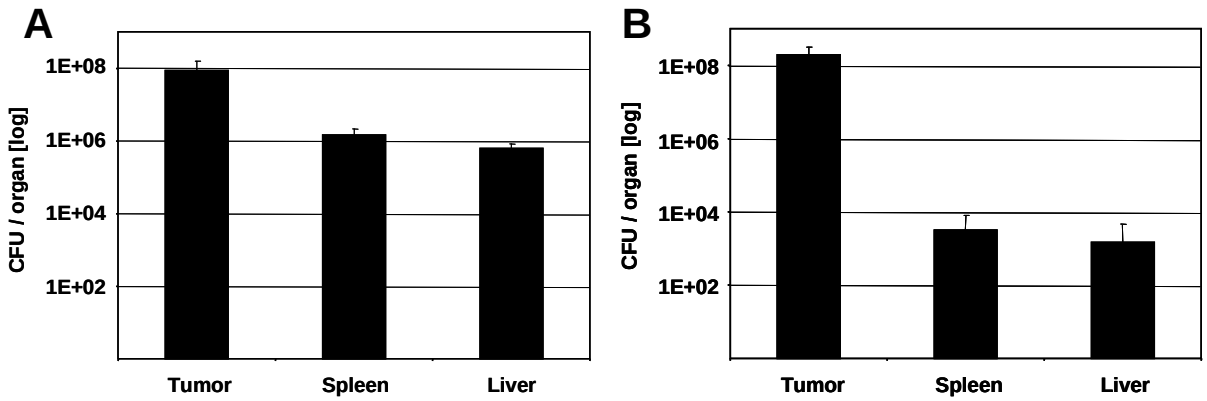



Fig. 3.7: Bacterial colonization in different tissues. Tumor bearing mice were i.v. infected with *S. typhimurium* SL7207 (**A**) and *E. coli* TOP10 (**B**), respectively. Two days p.i. tumor, spleen and liver were homogenized and plated and CFU per organ were determined. Results are representative for at least three independent experiments. Error bars show standard deviations.

The distribution of *E. coli* TOP10 in CT26 tumors two days after i.v. infection is generally comparable to the distribution of *S. typhimurium* SL7207. Like *Salmonellae*, *E. coli* were found only inside large necrotic areas, while no bacteria could be detected under the skin covering the solid tumors or inside viable tumor tissue. This is shown in Fig. 3.9. Fig. 3.9 A displays an overview of the infected tumor tissue and Fig. 3.9 B shows the border between viable (V) and necrotic (N) tumor tissue in enlargement. As for *S. flexneri*, the necrotic, loosely associated cells inside the necrosis were bordered by a region of densely packed cells with irregularly shaped nuclei. The *E. coli* were either found inside the necrosis or in close contact to those densely packed cells. Fig. 3.9 C is a magnification of the white box marked in Fig. 3.9 A and displays the bacteria inside the necrotic area. In Fig. 3.9 D, the white boxed part of Fig. 3.9 C is displayed in a higher magnification.

Comparing the distribution of *S. typhimurium* and *E. coli* to the distribution of *S. flexneri* inside solid CT26 tumors, it becomes clear that all tested bacterial strains accumulate preferably, if not exclusively, inside large necrotic areas (compare Fig. 3.22) leaving a rim of viable, non-infected tumor cells. The accumulation of bacteria inside the tumor was always associated with a macroscopic visible blackening of the surface of the subcutaneous tumors and the tumor growth was retarded or stopped. Sometimes, even shrinkage of the tumor could be observed. However, when the experiment was extended over the described two days, the viable cells of the tumor rim rapidly proliferated and the tumor started to regrow.

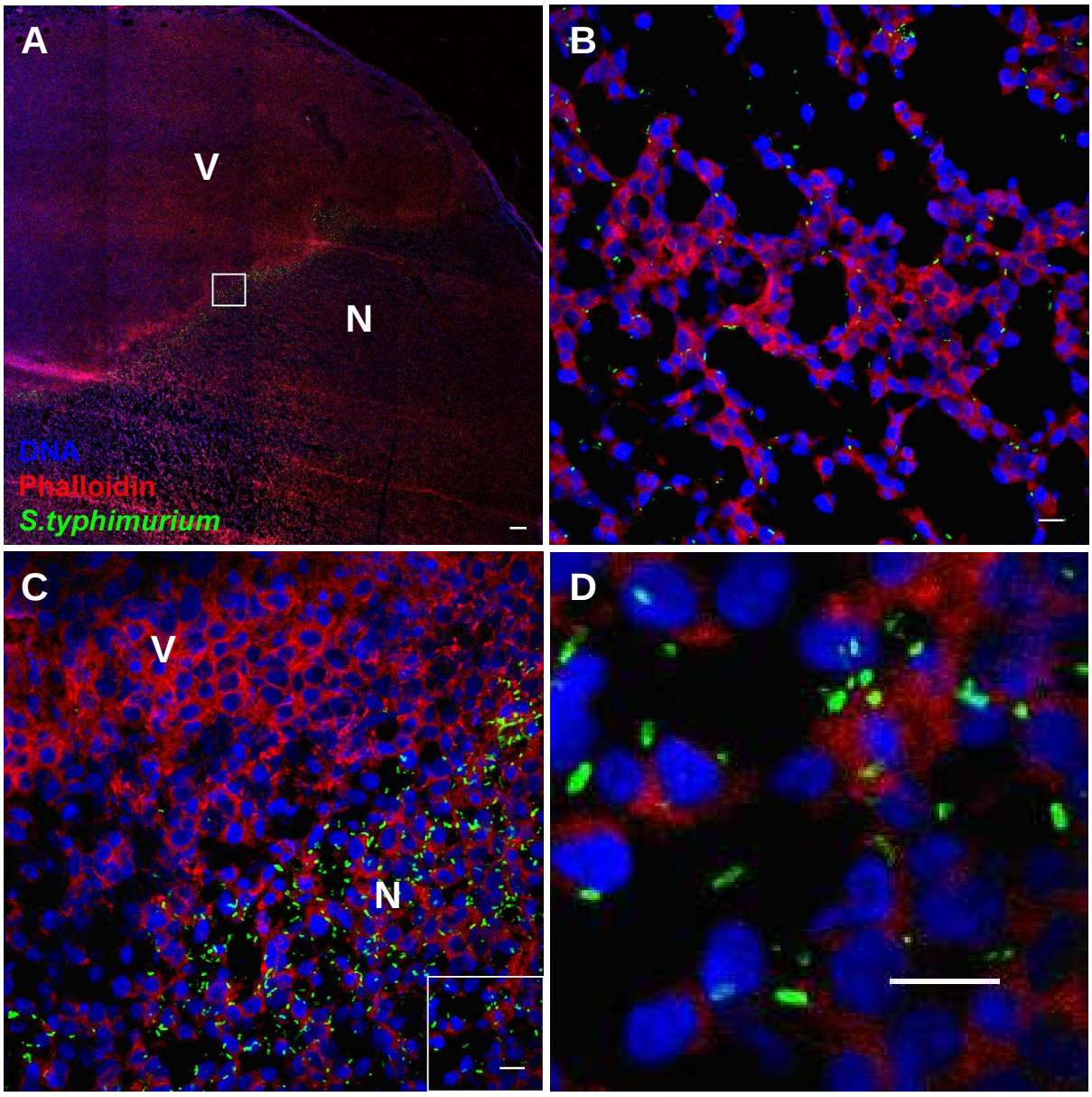



Fig. 3.8: Distribution of *S. typhimurium* SL7207 in solid CT26 tumors two days after intravenous infection. Bacteria are stained in green, DNA is stained in blue and the cellular actin is stained in red. (**A**) Low magnification overview of an *S. typhimurium* colonized tumor. (**B**) Higher magnification of the necrotic tumor tissue. (**C**) Higher magnification of the border between necrotic and vital tumor tissue. (**D**) High magnification of the bacteria-colonized, necrotic region. The white box in **A** indicates the area of enlargement in **C**, while the white box in **C** indicates the area of enlargement in **D**. The white bars represent 100 µm (**A**) and 10 µm (**B**, **C**, and **D**), respectively. The pictures are representative for at least three different *S. typhimurium* SL7207 infected CT26 tumors from independent experiments.

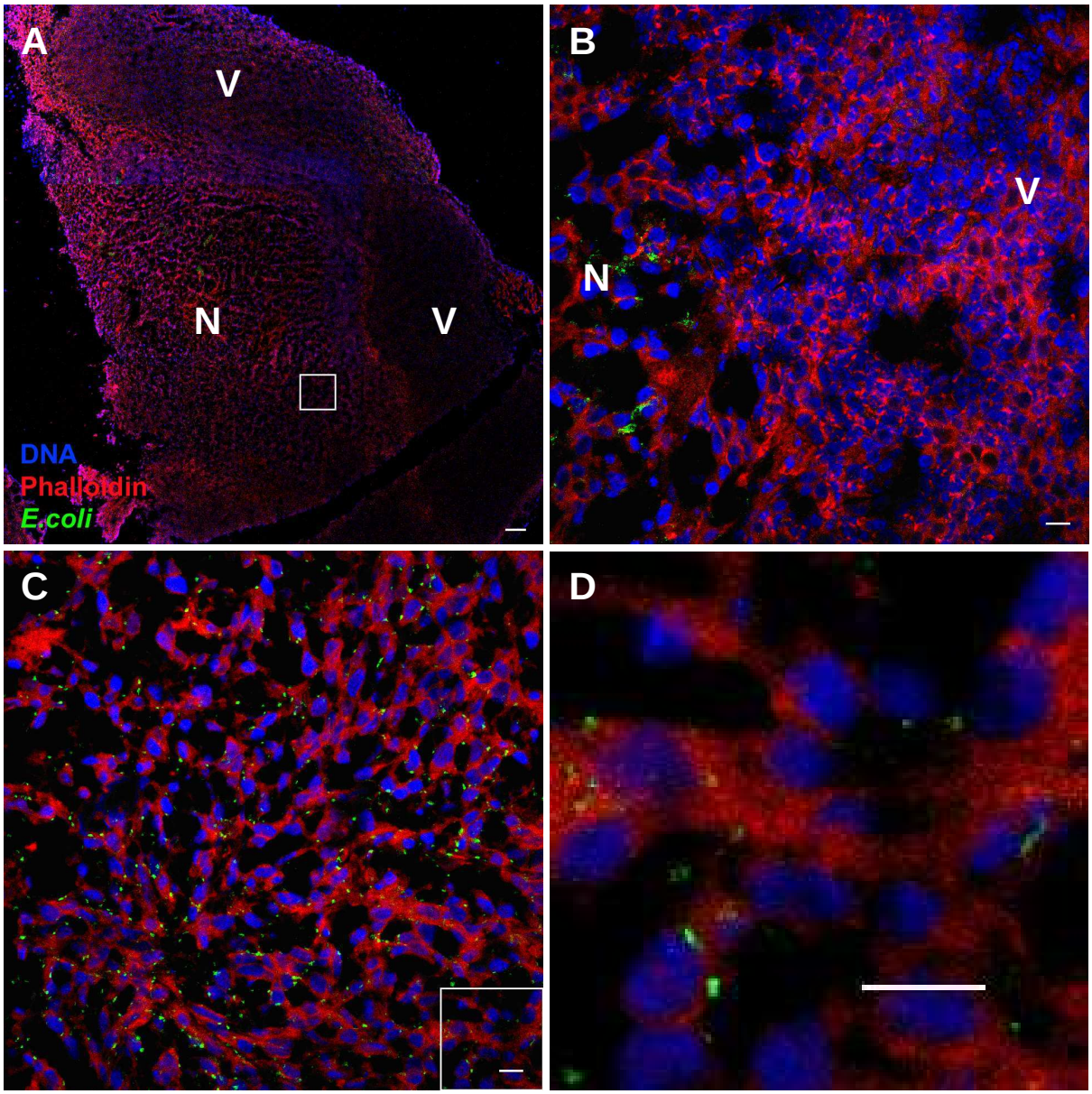



Fig. 3.9: Distribution of *E. coli* TOP10 in solid CT26 tumors two days after intravenous infection. Bacteria are stained in green, DNA is stained in blue and the cellular actin is stained in red. (**A**) Low magnification overview of an *E. coli* colonized tumor. (**B**) Higher magnification of the border between necrotic and vital tumor tissue. (**C**) Higher magnification of the necrotic tumor tissue. (**D**) High magnification of the bacteria-colonized, necrotic region. The white box in **A** indicates the area of enlargement in **C**, while the white box in **C** indicates the area of enlargement in **D**. The white bars represent 100 µm (**A**) and 10 µm (**B**, **C**, and **D**), respectively. The pictures are representative for at least three different *E. coli* TOP10 infected CT26 tumors from independent experiments.

Together, the depicted accumulation of the employed facultative anaerobic bacteria inside a large necrosis is independent of the route of infection. The infection itself cannot be sufficient to stop tumor growth since the bacteria are not able to kill all the viable, proliferating tumor cells.

## 3.4 Dramatic influx of neutrophils to the site of infection

All the bacteria strains tested so far were mainly restricted to the necrotic area although their growth characteristics should have allowed them to spread over the entire tumor. Hence, it was postulated that immune cells inside the tumor might be responsible for the bacterial distribution. To test this hypothesis, 10 µm cryosections of infected and uninfected tumors were stained with fluorescently conjugated antibodies against various immune cells. This revealed a tremendous influx of neutrophils to the site of infection.

Fig. 3.10 shows low magnification overviews of three different, uninfected control CT26 tumors. In all such tumors, neutrophilic granulocytes, which are double positive for the markers Gr1 and Mac1 and thus appear pink, are scattered as single cells or in small clusters all over the solid tumor. Sometimes, as can be seen in Fig. 3.10 C, the neutrophils appear accumulated in bigger clusters, usually at places of small necroses. All tumor cells seem to express low amounts of Mac1, as the whole tumor is faintly stained blue. The Mac1 staining is more intense at the rim of the solid tumors. In general it is known that Mac1 can be expressed on macrophages, granulocytes and on dendritic cells.

Two days after infection neutrophils had invaded the tumor in large numbers. They are located at the border between the “healthy” tumor tissue at the outer rim and necrosis that is populated by the bacteria at the inner rim (Fig. 3.11, Fig. 3.12, Fig 3.13 and Fig. 3.14). The necrotic part of the tumor is neither stained by Gr1 nor Mac1. Here, most of the bacteria can be found. This necrotic area is bordered by a thick layer of Gr1/Mac1 double positive neutrophils, the majority of which is in close contact with bacteria. The vital tumor tissue that is neighboring the neutrophil border is positive for Mac1. This could be due to an infiltration of Mac1-positive macrophages, but also because of the low expression of Mac1 on the vital CT26 cells.

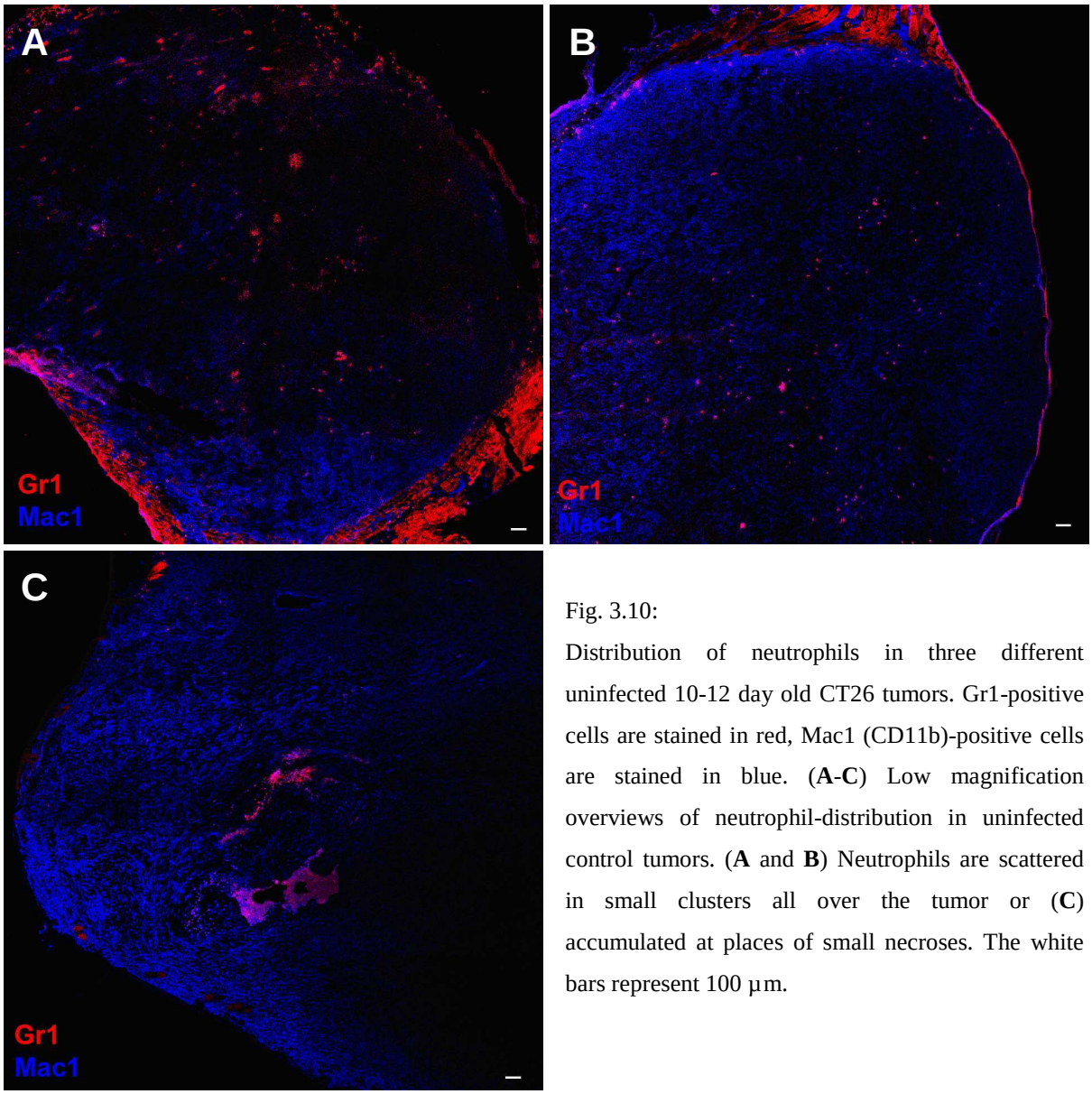



Fig. 3.10:
Distribution of neutrophils in three different uninfected 10-12 day old CT26 tumors. Gr1-positive cells are stained in red, Mac1 (CD11b)-positive cells are stained in blue. (**A**-**C**) Low magnification overviews of neutrophil-distribution in uninfected control tumors. (**A** and **B**) Neutrophils are scattered in small clusters all over the tumor or (**C**) accumulated at places of small necroses. The white bars represent 100 µm.

For *S. flexneri* Δdap (Fig. 3.10), the restriction to the necrotic tissue is not as strict as for *S. typhimurium* (Fig. 3.12) and *E. coli* (Fig. 3.13). As can be seen in Fig. 3.11 E, *S. flexneri* could sometimes be found in viable tumor tissue. As proposed before, the occurrence of *S. flexneri* in vital tumor tissue might – at least in part – be due to the different infection route. Even here, the bacteria are in close contact with neutrophils.

*S. flexneri* can also be applied systemically. Since they do not represent a pathogen in the mouse, only few bacteria survive after administration. Nevertheless, some of them can be found in the tumor, although they do not have a strong intrinsic tumor-targeting ability (Zelmer, 2005). Interestingly, the *Shigellae* found in tumors after systemic application are also located inside necrotic areas (Fig. 3.12). In these cases, the bacteria-containing necroses are

surrounded by numerous neutrophils that migrate into the tumor upon invasion, similar to intratumoral application of *Shigellae*.

Contrary to i.t. injected *S. flexneri*, *S. typhimurium* SL7207 was strictly found inside the necrosis (Fig. 3.13 A-C) or associated with neutrophils bordering the necrosis (Fig. 3.13 C and D). No *Salmonellae* could be detected in vital tumor tissue. Similar results were obtained for *E. coli* TOP10. No *E. coli* were found in viable tumor tissue, while most bacteria had disseminated inside the large necrosis (Fig. 3.14 D).

Together, the cellular arrangement inside bacteria-colonized tumors suggests that the microorganisms are kept from spreading homogenously over the solid tumor by neutrophils that are attracted after bacterial colonization and migrate towards the necrosis not only to remove the dead, necrotic cells but also to inhibit the bacteria from further spreading into healthy tissue. The inhibition of bacterial distribution into vital tumor tissue might be the reason for the low gene transfer efficiency with *S. flexneri* Δdap in solid CT26 tumors observed before.

To investigate whether *Shigella*-mediated gene transfer could be observed in cryosections of CT26 tumors that were colonized by *S. flexneri* Δdap carrying the reporter plasmid pCMVluc-λ2In-m2A, 10 µm cryosections of these tumors were stained with antibodies directed against luciferase, the reporter protein. In addition, the tumor sections were stained with antibodies directed against *Shigellae* and against Gr1-positive cells in order to localize bacteria, neutrophils and necrosis.

As can be seen in Figure 3.15, the majority of cells did not express luciferase. Only very few cells are brightly stained for the enzyme. These luciferase expressing cells are magnified in 3.15 B and 3.15 C. According to the enlarged images, luciferase expressing cells (marked by arrow heads) were in areas, in which bacteria (marked by arrows) and neutrophils are located. Such rare luciferase expressing cells were only found in vital tumor tissue (**V**), distant from the large necrosis (**N**). The number of luciferase expressing cells per section was very scarce. This might explain the failure of detecting luciferase activity reproducibly in tumor homogenates.

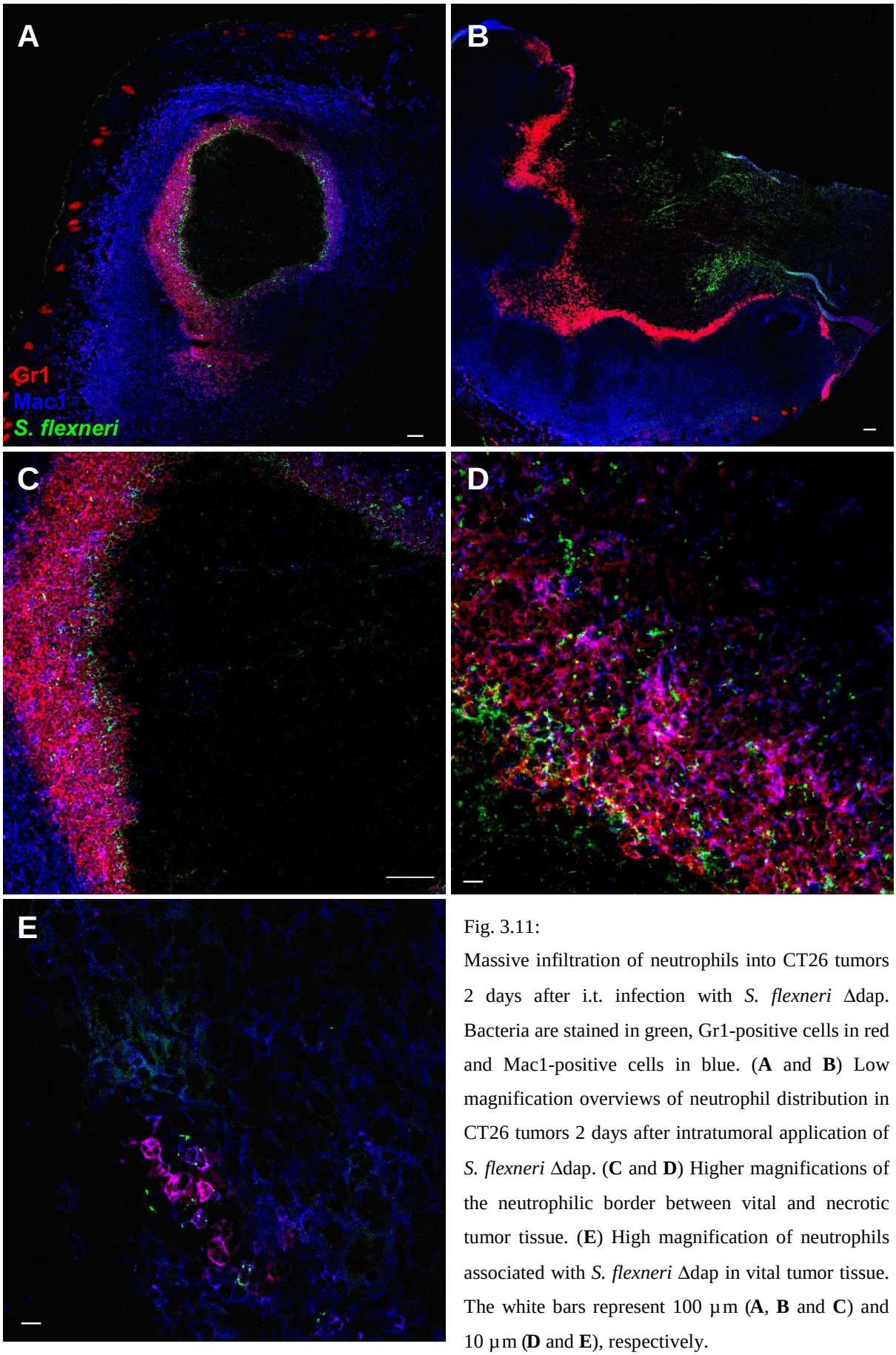



Fig. 3.11:
Massive infiltration of neutrophils into CT26 tumors 2 days after i.t. infection with *S. flexneri* Δdap. Bacteria are stained in green, Gr1-positive cells in red and Mac1-positive cells in blue. (**A** and **B**) Low magnification overviews of neutrophil distribution in CT26 tumors 2 days after intratumoral application of *S. flexneri* Δdap. (**C** and **D**) Higher magnifications of the neutrophilic border between vital and necrotic tumor tissue. (**E**) High magnification of neutrophils associated with *S. flexneri* Δdap in vital tumor tissue. The white bars represent 100 µm (**A**, **B** and **C**) and 10 µm (**D** and **E**), respectively.

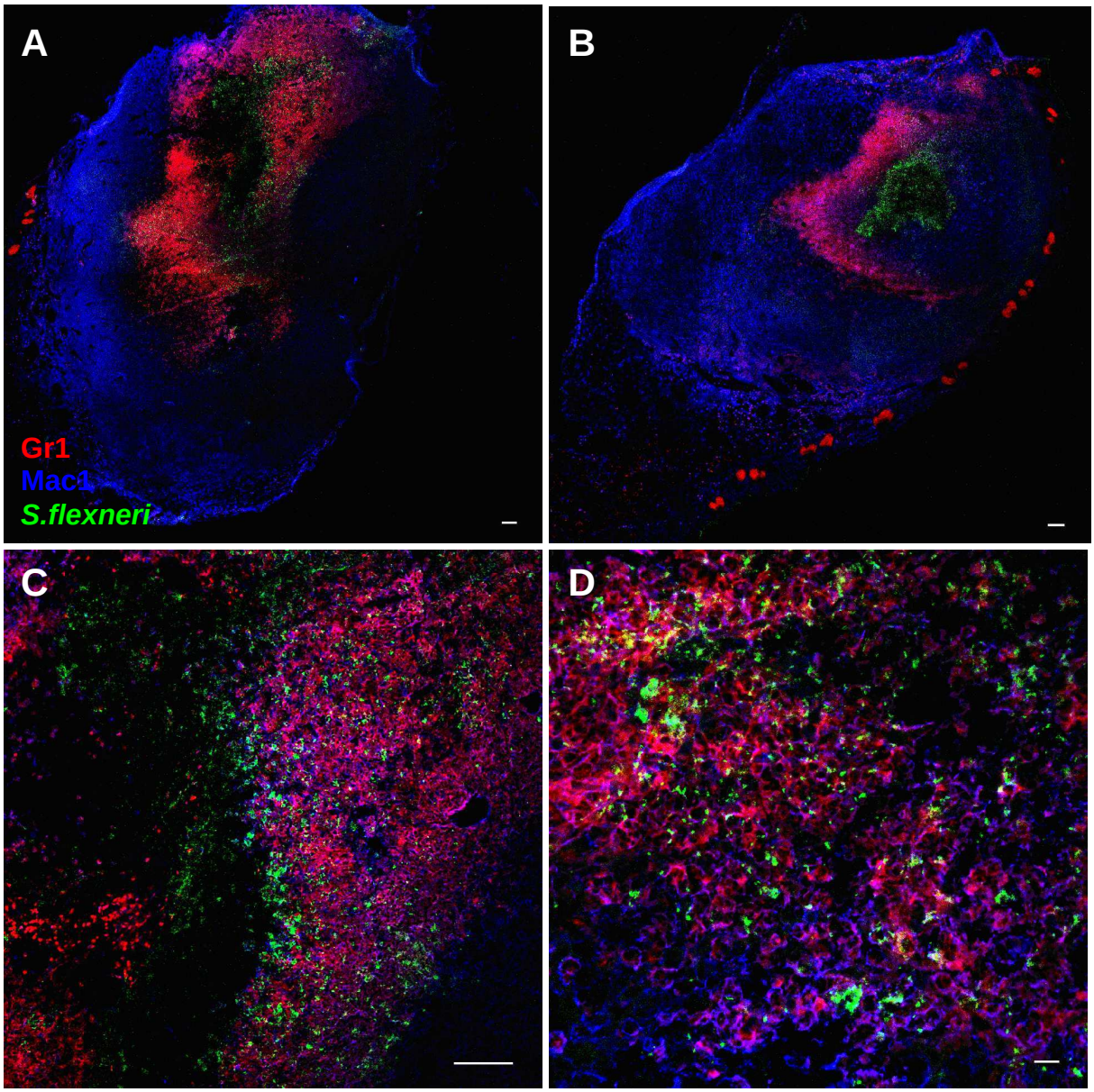



Fig. 3.12: Massive infiltration of neutrophils into CT26 tumors two days after intravenous infection with *S. flexneri* Δdap. Bacteria are stained in green, Gr1-positive cells are stained in red and Mac1-positive cells are stained in blue. (**A** and **B**) Low magnification overviews of neutrophil distribution in CT26 tumors 2 days after intravenous infection with *S. flexneri* Δdap. (**C** and **D**) Higher magnification of the neutrophilic border between vital and necrotic tumor tissue (**D**). The white bars represent 100 µm (**A-C**) and 10 µm (**D**), respectively. The pictures are representative for at least three different tumors from independent experiments.

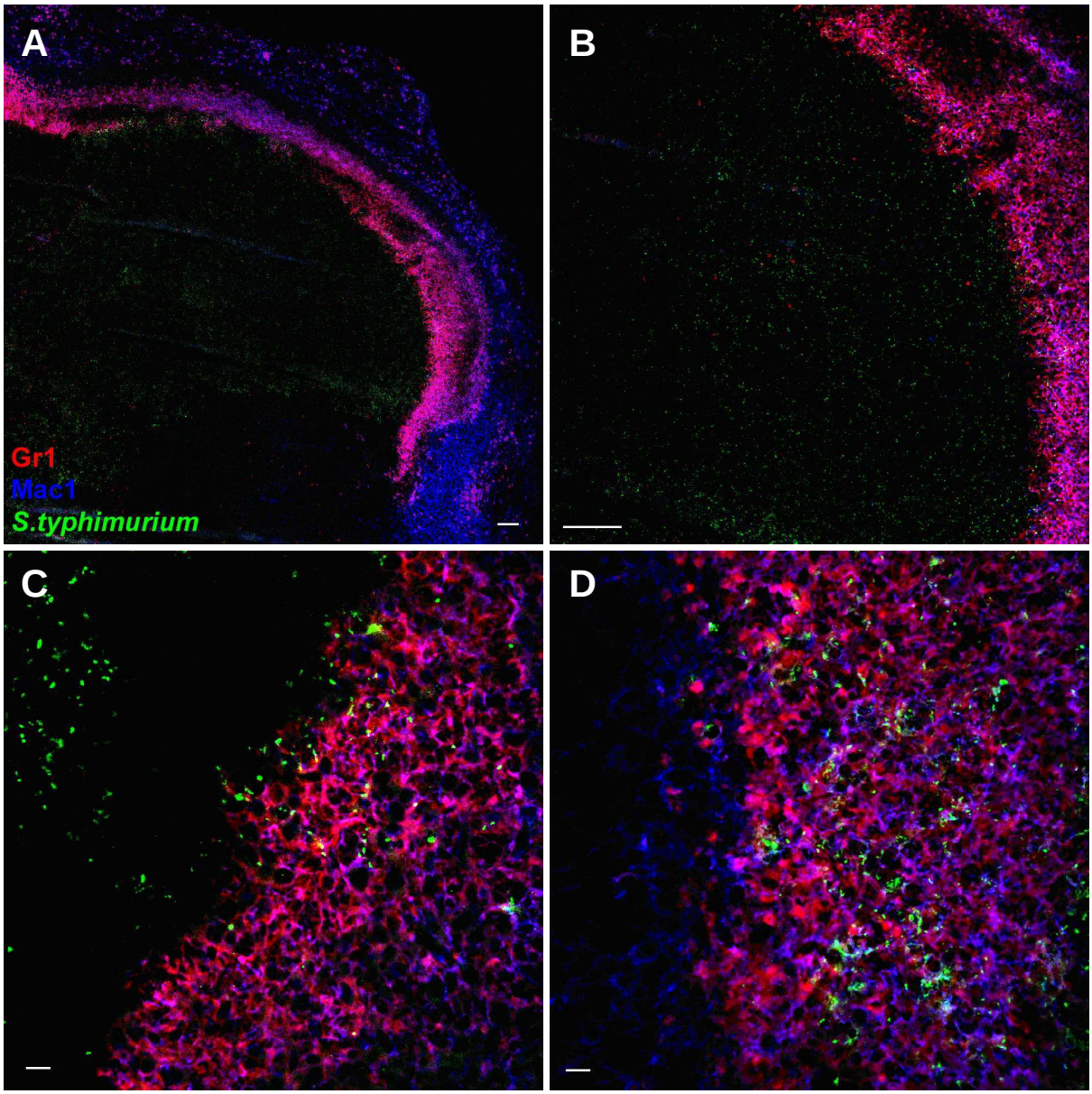



Fig. 3.13: Massive infiltration of neutrophils into CT26 tumors two days after intravenous infection with *S. typhimurium* SL7207. Bacteria are stained in green, Gr1-positive cells are stained in red and Mac1-positive cells are stained in blue. (**A**) Low magnification overview of neutrophil distribution in CT26 tumors 2 days after intravenous infection with *S. typhimurium* SL7207. (**B**-**D**) Higher magnifications of the neutrophilic border between vital and necrotic tumor tissue. The white bars represent 100 µm (**A** and **B**) and 10 µm (**D** and **E**), respectively. The pictures are representative for at least three different tumors from independent experiments.

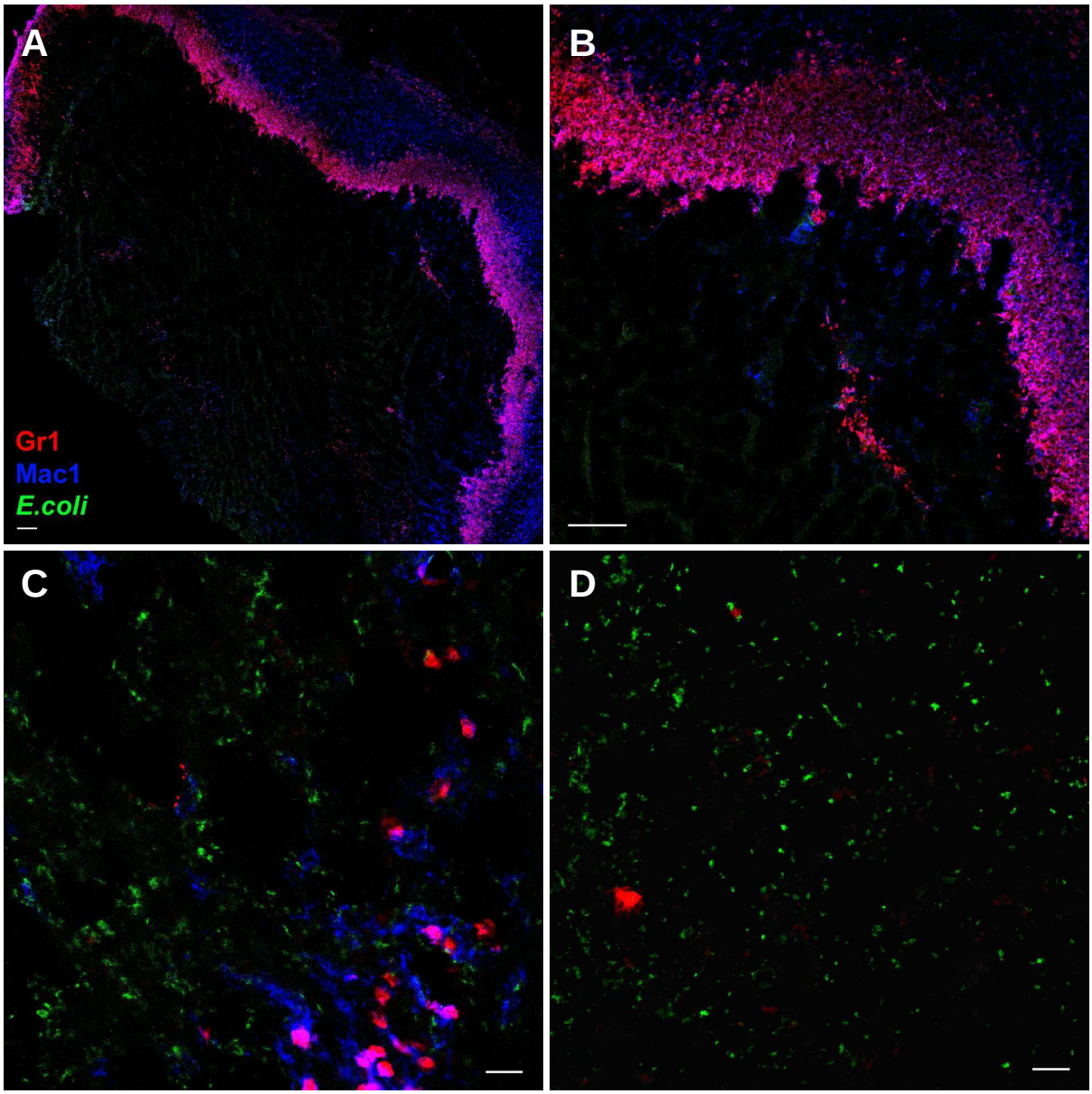



Fig. 3.14: Massive infiltration of neutrophils into CT26 tumors two days after intravenous infection with *E. coli* TOP10. Bacteria are stained in green, Gr1-positive cells are stained in red and Mac1-positive cells are stained in blue. (**A**) Low magnification overview of neutrophil distribution in CT26 tumors 2 days after intravenous infection with *E. coli* TOP10. (**B** and **C**) Higher magnifications of the neutrophilic border between vital and necrotic tumor tissue. (**D**) High magnification of *E. coli* TOP10 inside the necrosis. The white bars represent 100 µm (**A** and **B**) and 10 µm (**D** and **E**), respectively. The pictures are representative for at least three different tumors from independent experiments.

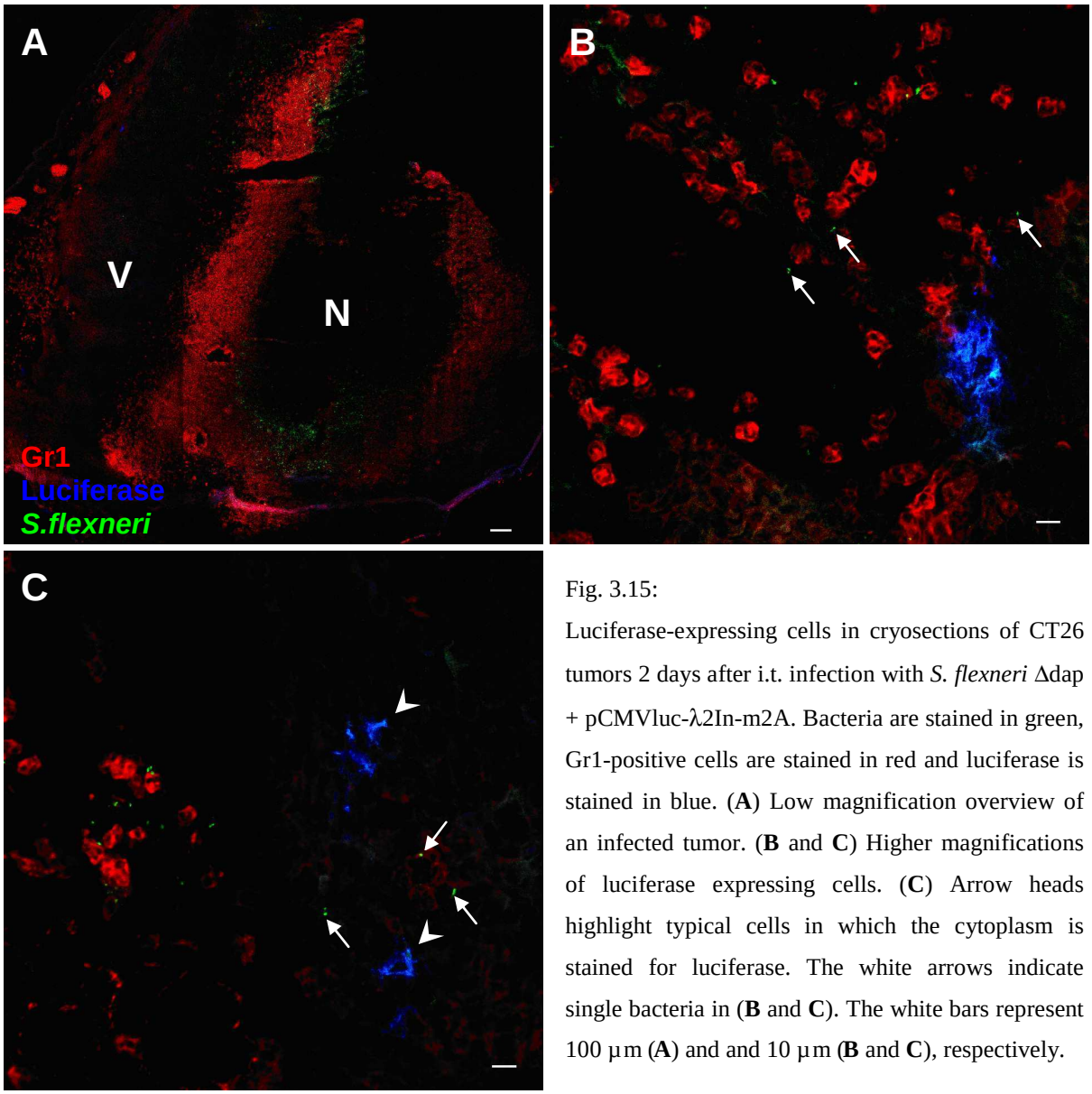



Fig. 3.15:
Luciferase-expressing cells in cryosections of CT26 tumors 2 days after i.t. infection with *S. flexneri* Δdap + pCMVluc-λ2In-m2A. Bacteria are stained in green, Gr1-positive cells are stained in red and luciferase is stained in blue. (**A**) Low magnification overview of an infected tumor. (**B** and **C**) Higher magnifications of luciferase expressing cells. (**C**) Arrow heads highlight typical cells in which the cytoplasm is stained for luciferase. The white arrows indicate single bacteria in (**B** and **C**). The white bars represent 100 µm (**A**) and and 10 µm (**B** and **C**), respectively.

As depicted in Fig. 3.11-3.14, the bulk of bacteria were enclosed by neutrophils inside a large necrosis, associated with dead, necrotic cells into which gene transfer is not possible. The fact that most of the *Shigellae* were accumulated inside the necrosis and only few bacteria were found in vital tumor tissue is very likely one cause for the low bacterial gene transfer efficiency *in vivo*. By extrapolation, this might also apply to other bacterial transfer systems like *L. monocytogenes*, where similar failure of gene transfer was accounted (Zelmer, 2005).

Generally, in an ideal bacteria-mediated tumor therapy, the bacteria should be homogenously distributed over the tumor and reach all cells of a tumor, including well oxygenated, vital cells but also hypoxic and necrotic areas. In this respect, the “border of neutrophils” might be keeping the bacteria from homogeneous dissemination throughout the solid tumors. Therefore, the depletion of neutrophils or the prevention of an infiltration of neutrophils

towards the infection should improve bacterial dissemination inside solid CT26 tumors and might enhance gene transfer efficiency.

## 3.5 Depletion of host neutrophils

The hypothesis that the neutrophil border has an impact on the spreading of the bacteria throughout the tumor and their invasion into viable tumor tissue was tested by depleting neutrophils from the mice. This was accomplished by triple i.p. injections of 25 µg and 100 µg anti-Gr1 antibody, respectively. The antibody was injected 1 day before bacterial infection (-1), simultaneously with infection (0) and 1 day after infection (1). All mice survived the antibody-treatment and the concomitant infection without obvious impairments in health. Two days after infection the efficiency of the depletion was controlled via flow cytometry of blood of treated and control mice. As can be seen in Fig. 3.16, a triple treatment with 25 µg anti-Gr1 was sufficient to reduce the number of neutrophils in the blood to 4% of the number found in untreated control mice. A higher dosage of the antibody resulted only in slight enhancement of the depletion (down to 3%). Thus, all further experiments were performed with a triple dose of 25 µg anti-Gr1.

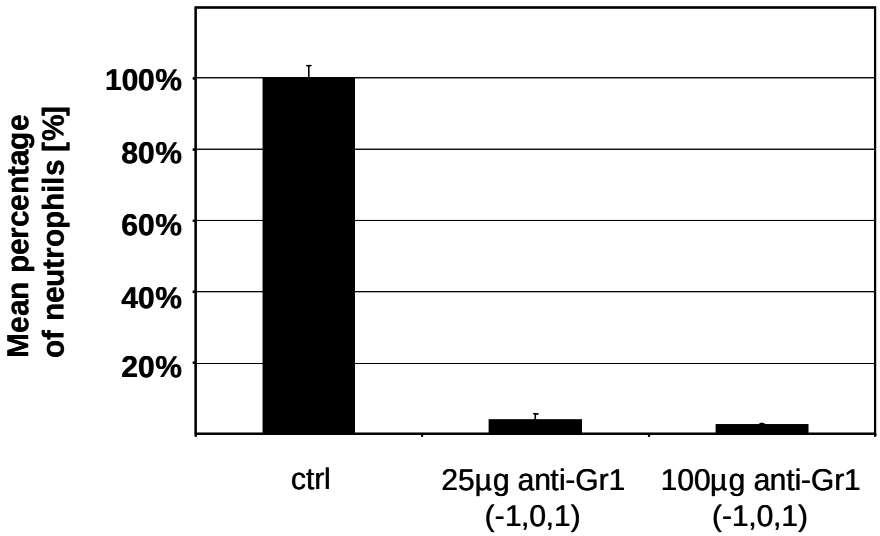



Fig. 3.16: Neutrophil depletion in the blood of BALB/c mice. Neutrophils were depleted by triple i.p. injections of 25 µg and 100 µg anti-Gr1, respectively, one daybefore (-1), simultaneously (0) and one day after (-1) infection. Two days post infection, blood samples were taken and analyzed by flow cytometry. The percentage of neutrophils in the blood of neutrophil depleted mice was normalized to the percentage of neutrophils in non-depleted tumor bearing control mice.

### 3.5.1 Stronger accumulation of bacteria inside the tumor after depletion of neutrophils

Two days after infection tumor-bearing mice that were either depleted of neutrophils or not were sacrificed and the CFU per tissue were determined (Fig. 3.17 A-C). To ensure successful depletion of neutrophils in every experiment, the depletion was controlled via flow cytometry of blood, as described above. As displayed in Fig. 3.17 A, the depletion of neutrophils lead to a colonization of *S. typhimurium* in all tested tissues that was approximately 4-5 times higher than in non-depleted, infected control mice. Thus, a depletion of neutrophils has a strong influence on bacterial colonization of solid tumors. However, at the same time the bacterial numbers in all other organs tested was also increased, resulting in a higher total bacterial burden of the mice.

Regarding *E. coli* TOP10 (Fig. 3.17 B), this effect was more diversified. Here, the number of bacteria inside the tumor was about 7 times higher in depleted compared to non-depleted mice. In contrast, the other organs were only slightly more affected in depleted mice, if at all, compared to non-depleted mice. Thus, *E. coli* primarily leads to higher bacterial numbers in the tumor without a concomitant increased bacterial burden in the other organs.

*S. flexneri* Δdap (Fig. 3.17 C) basically show the same pattern as *S. typhimurium* and *E. coli* although the differences between the bacterial load inside the tumor of neutrophil-depleted in comparison to non-depleted mice are not as distinct. This might, again, be due to the way of bacterial administration. The small volume of the i.t. application might render this route of infection more error prone than i.v. infections. Small deviations of the intended injection volume will result in significant alterations in bacterial numbers in the tumor two days later. Therefore, the effect of the neutrophil depletion on the CFU in tumors might be obscured by the large standard deviation in non-depleted mice.

Different to the intratumoral colonization with *S. flexneri* Δdap, the number of *Shigellae* in other organs seems not to be influenced by the depletion. As mentioned before (chapter 3.1.4), *S. flexneri* are able to spread from the tumor into distant organs. This spreading is obviously not affected by the presence or absence of neutrophilic granulocytes.

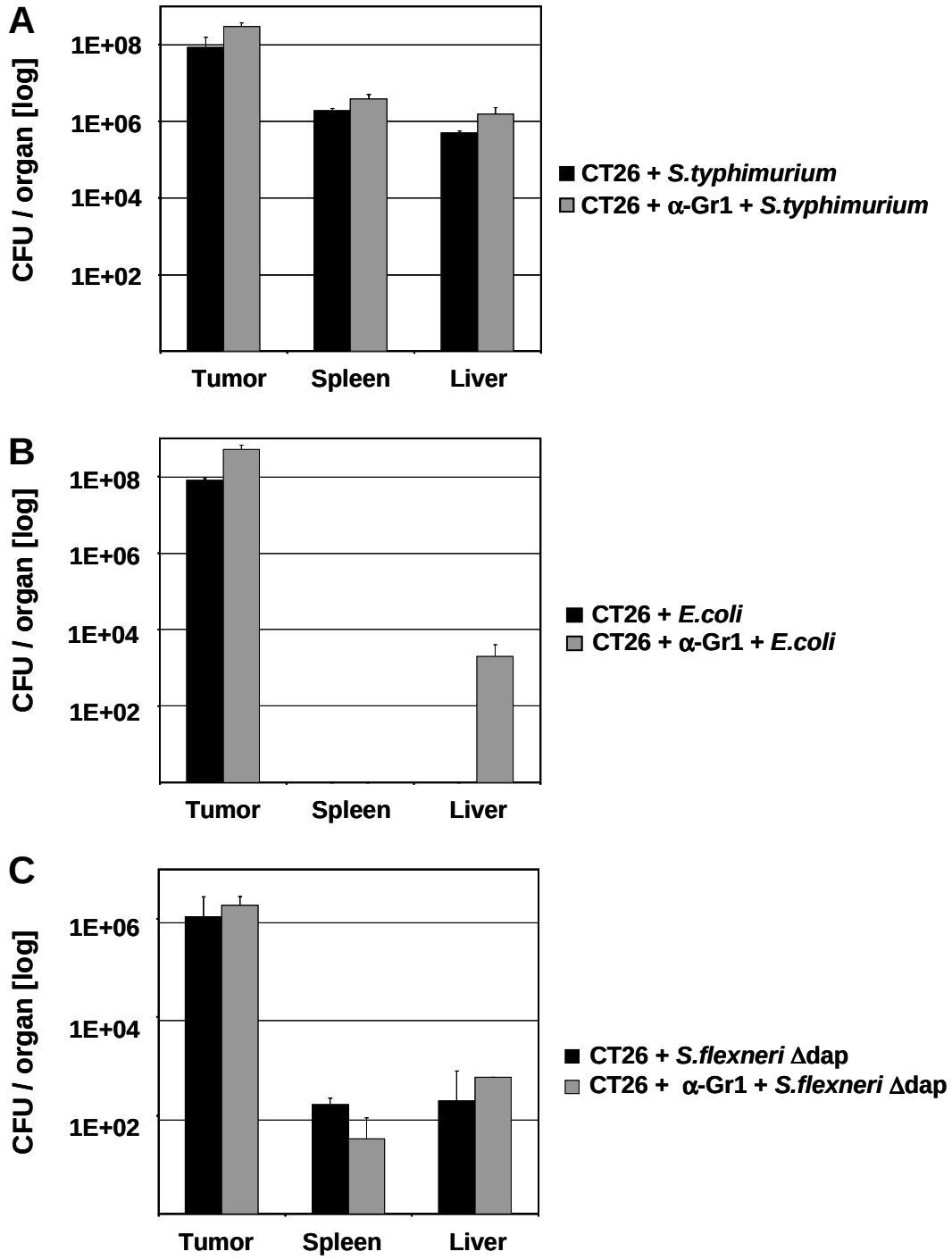



Fig. 3.17: Bacterial accumulation in different tissues with or without neutrophil depletion. Tumor-bearing, anti-Gr1 treated and non-treated mice were infected i.v. with *S. typhimurium* SL7207 (**A**), *E. coli* TOP10 (**B**) or infected i.t. with *S. flexneri* Δdap (**C**). Neutrophils were depleted by triple i.p. injections of 25 µg anti-Gr1, on day -1, 0 and 1. Two days post infection, tumor, spleen and liver were homogenized and plated and the CFU per tissue were determined. The black bars represent the CFUs in non-depleted mice while the grey bars represent the CFUs in neutrophil-depleted, infected mice. The error bars show standard deviations. Results are representative for at least 2 independent experiments with 3-5 mice.

### 3.5.2 Bacteria are found in vital tumor tissue of neutrophil-depleted mice

In parallel to plating tissue homogenates, tumors from neutrophil-depleted mice were snapfrozen and analyzed by histology, as before. Fig. 3.18-3.19 demonstrate that most of the Gr1/Mac1 double-positive neutrophils in the tumor were depleted, as intended. Remarkably, under these conditions all bacteria tested were able to disseminate into vital tumor tissue (Fig. 3.18 D, Fig. 3.19 D, Fig. 3.20 D). However, the majority of bacteria still remained inside the necrosis.

Fig. 3.18 A and Fig. 3.18 B show low magnification overviews of *S. typhimurium*-infected, neutrophil-depleted, CT26 tumors two days p.i.. In both experiments, few neutrophils remained that were clustered in a small layer. Obviously, such few neutrophils were not sufficient to keep the bacteria from spreading into vital tumor tissue, as *Salmonellae* could be found on both sites of this neutrophil border. In Fig. 3.18 B *S. typhimurium* appear to migrate around the few remaining neutrophils. Fig. 3.18 C shows an enlargement of the remaining neutrophil border and of *Salmonellae* settling on both sites of neutrophils. Fig. 3.19 D highlights *Salmonellae* inside vital tumor tissue.

In the overviews of neutrophil-depleted, *E. coli* TOP10-infected tumors (Fig. 3.19 A and Fig. 3.19 B) no such remaining neutrophil border could be observed. Obviously the treatment was more efficient in this case. Fig. 3.19 C shows a higher magnification of *E. coli* inside the necrosis. One can clearly see the destruction of the necrotic tissue. The necrotic cells stick to each other and appear to form a mesh of sticky necrotic cells. Up to now it is unclear, whether this is caused by the cutting process, or whether the mesh-like structure is also found in the intact tumor. However, it is obvious that the cells in this structure are dead. Fig. 3.19 D highlights vital tumor tissue. Contrary to Fig. 3.19 C, the cells inside vital tissue are interconnected with each other and form a smooth layer of cells. Similar to the results obtained for *S. typhimurium* in neutrophil-depleted mice, bacteria can now be found inside vital tumor tissue. Single *E. coli* are marked with white arrows.

The distribution of *S. flexneri* Δdap (Fig. 3.20) in neutrophil-depleted CT26 tumors two days after infection resembles the distribution of *S. typhimurium* and *E. coli*. However, in contrast to these other strains, the structure of *S. flexneri* Δdap infected, neutrophil-depleted tumors seems to be more amorphic. Differences between vital and necrotic tumor tissue are hardly visible and the majority of the tumor appears to be necrotic. In Fig. 3.20 C the transition from highly colonized, clearly necrotic tumor tissue to less colonized, vital tumor tissue is enlarged. Fig. 3.20 D finally shows single *S. flexneri* in tumor tissue that is mostly vital. The bacteria are marked with white arrows.

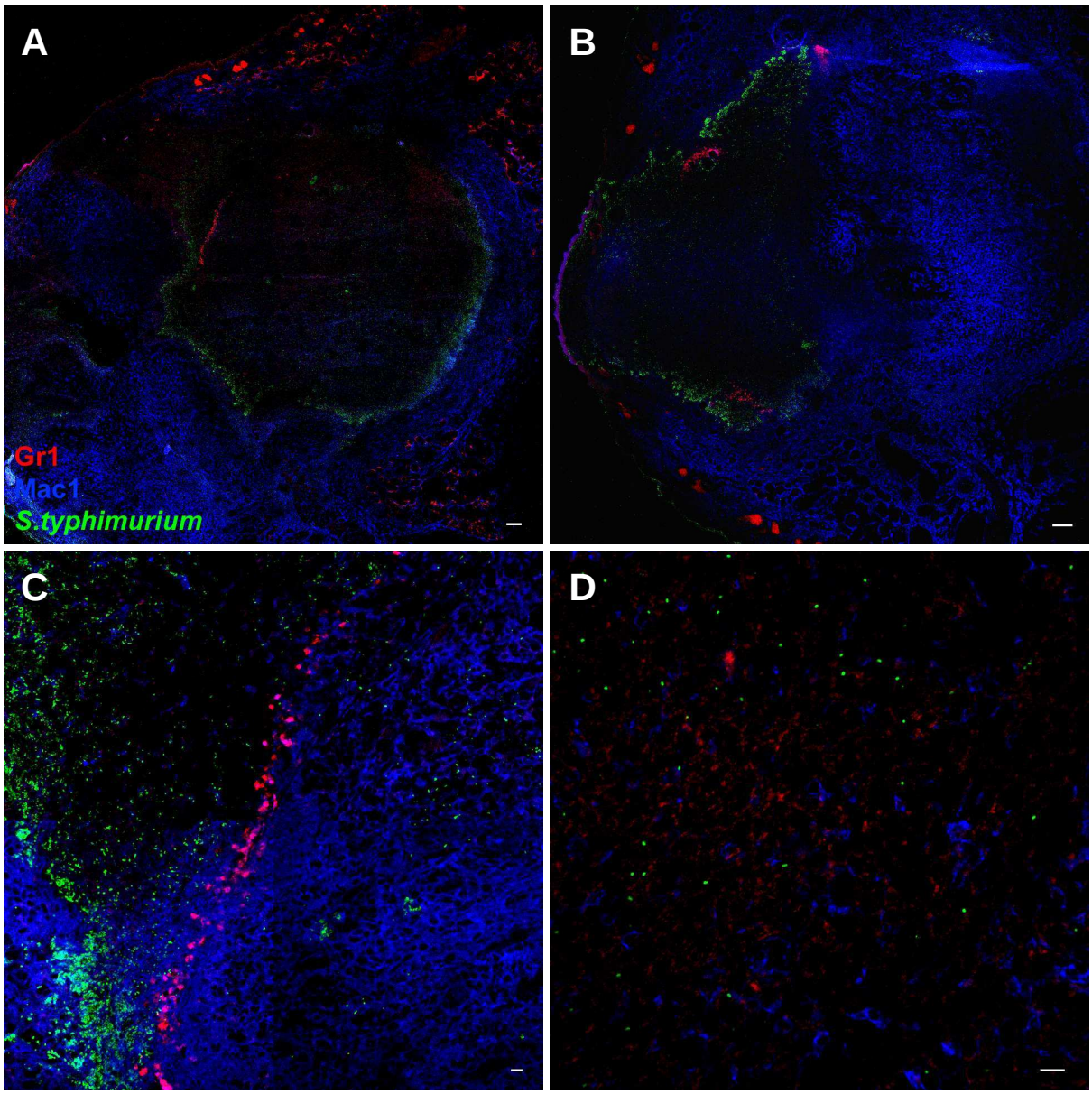



Fig. 3.18: Cryosections of CT26 tumors from *S. typhimurium* SL7207-infected, neutrophil-depleted mice were prepared two days p.i. Gr1-positive cells are stained in red, Mac1-positive cells are stained in blue and bacteria are stained in green. (**A**) and (**B**) Low magnification overviews; a small border of neutrophils can still be seen, which is apparently bypassed by the bacteria. (**C**) Higher magnification of the neutrophil border that remained. Bacteria can be seen on both sides of the neutrophils. (**D**) High magnification of *S. typhimurium* in vital tumor tissue. The white bars correspond to 100 µm in **A** and **B** and to 10 µm in **C** and **D**, respectively. Pictures are representative for 3-5 tumors from at least two independent experiments.

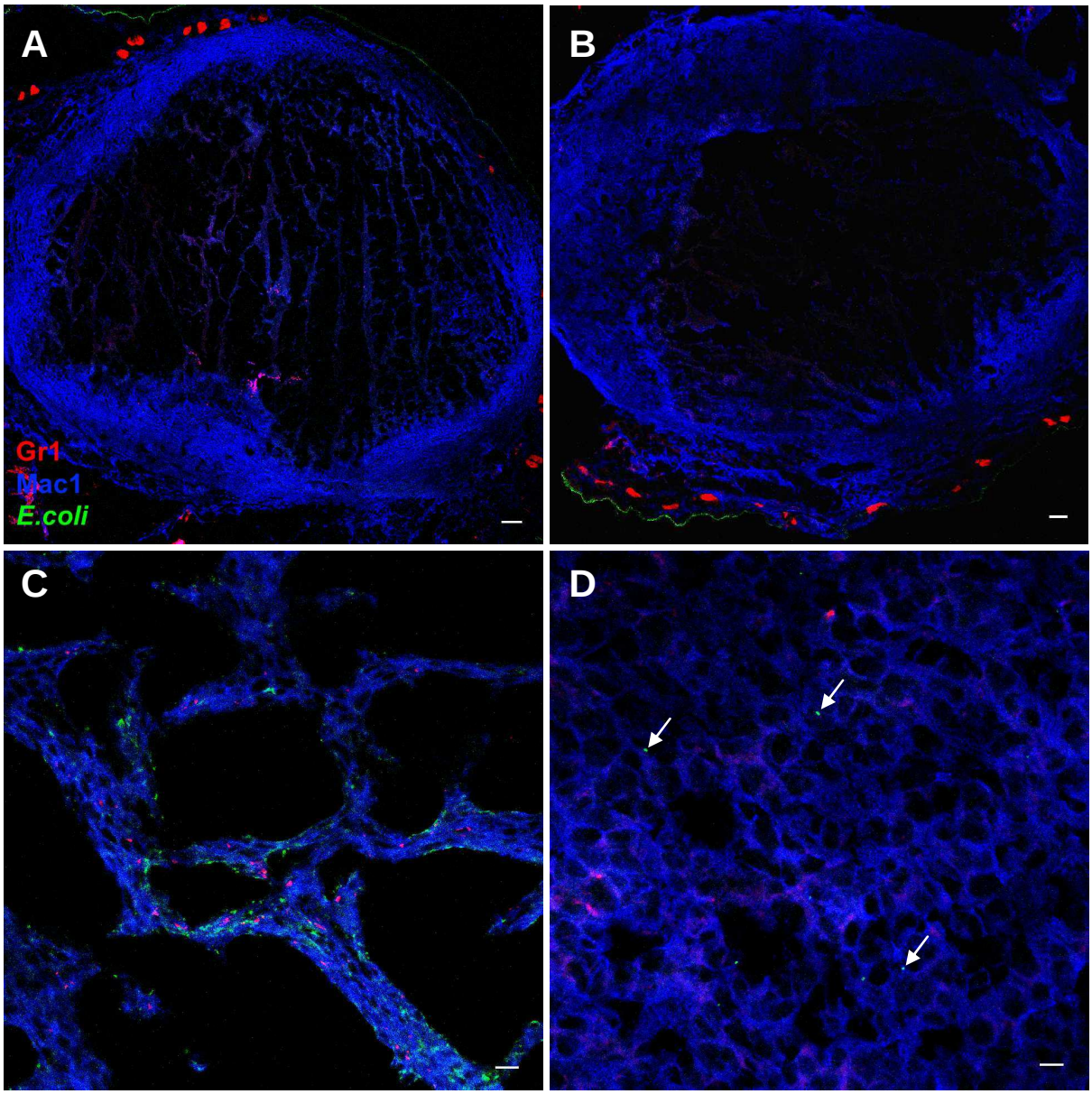



Fig. 3.19: Cryosections of CT26 tumors from *E. coli* TOP10-infected, neutrophil-depleted mice were prepared two days p.i. Gr1-positive cells are stained in red, Mac1-positive cells are stained in blue and bacteria are stained in green. (**A**) and (**B**) Low magnification overviews; almost no neutrophils can be detected inside the tumor. (**C**) High magnification of bacteria inside the necrosis. (**D**) High magnification of *E. coli* in vital tumor tissue. Some single bacteria are indicated by white arrows. The white bars correspond to 100 µm in **A** and **B** and to 10 µm in **C** and **D**, respectively. Pictures are representative for 3-5 tumors from at least two independent experiments.

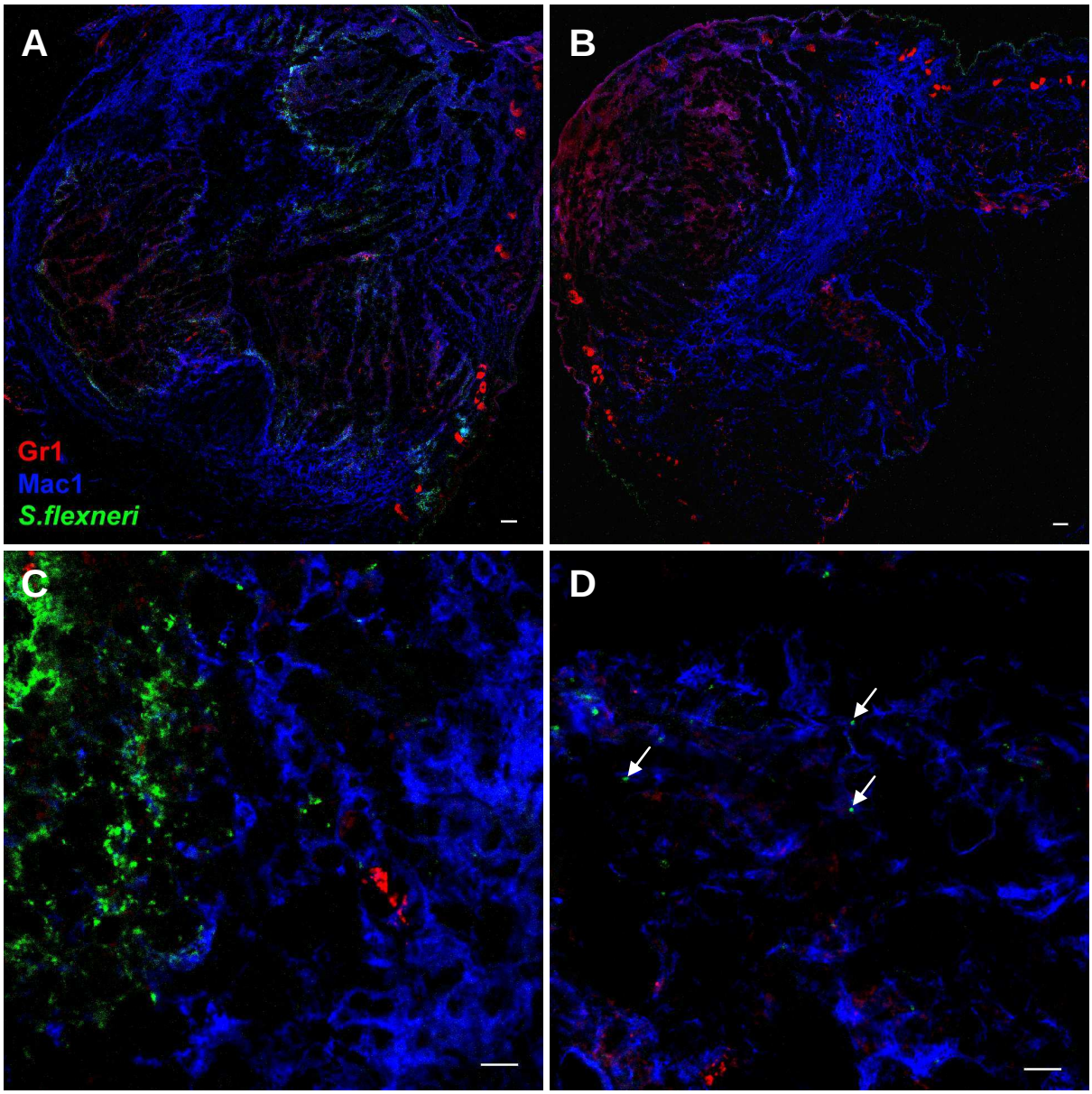



Fig. 3.20: Cryosections of CT26 tumors from *S. flexneri* Δdap-infected, neutrophil-depleted mice were prepared two days p.i. Gr1-positive cells are stained in red, Mac1-positive cells are stained in blue and bacteria are stained in green. (**A** and **B**) Low magnification overviews; almost no neutrophils can be detected inside the tumor. The tumor architecture has become less structured. Differentiation between viable and necrotic tumor tissue has become very difficult. (**C**) High magnification of the border between necrotic and vital tumor tissue. (**D**) High magnification of *S. flexneri* in mostly vital tumor tissue. Some single bacteria are indicated by white arrows. The white bars correspond to 100 µm in **A** and **B** and to 10 µm in **C** and **D**, respectively. Pictures are representative for 3-5 tumors from at least two independent experiments.

Taken together, the depletion of neutrophils via triple injections of anti-Gr1 leads to an increased dissemination of all bacterial strains tested inside solid CT26 tumors and most impressively, to a spreading of the bacteria into vital tumor tissue.

### 3.5.3 Depletion of neutrophils leads to increase of necrosis

Analysis of the cryosections under the various conditions described above had also suggested that the size of necrosis was increased in bacteria-colonized tumors of depleted mice. To determine the exact size of necrosis under various conditions, tumors were removed and fixed in paraformaldehyde. The fixed tumors were embedded in paraffin, cut into 5 µm sections and stained with hematoxylin and eosin. The size of the necrosis of a tumor was evaluated by examining every fifth section of the tumor from different locations i.e. from the center or the rim. This was accomplished with the cell^D software from Olympus, which allows the calculation of marked areas inside a histological section. The normalized results are displayed in Table 3.1. According to these evaluations, the size of necrosis clearly increased from around 10% in non-infected tumors to up to 60%-70% in infected tumors. After depletion of neutrophils, the size of necrosis increased even further and could reach to up to 90% of the entire tumor tissue. Increase in the size of necrosis is slightly more developed in *S. flexneri*-colonized tumors in comparison to tumors that were infected with the two other bacteria. The cause of this difference between the single bacterial strains is presently unclear. Figures 3.21-3.23 show representative data Table 3.1 is based upon.

Table 3.1: Size of necrosis in infected and uninfected CT26 tumors with and without depletion of neutrophils

| Tumor | Bacteria | anti-Gr1 | Percentage Necrosis |
|---|---|---|---|
| CT26 | Not infected | – | 5-15% |
| | *S. typhimurium* SL7207 | – | 60-65% |
| | *S. typhimurium* SL7207 | + | 75-85% |
| | *E. coli* TOP10 | – | 60-65% |
| | *E. coli* TOP10 | + | 80-85% |
| | *S. flexneri* Δdap | – | 65-70% |
| | *S. flexneri* Δdap | + | 85-90% |

The increase of necrosis two days after infection with any of the three bacterial strains in CT26 tumors can be seen when comparing Fig. 3.21 and Fig. 3.22. Necrosis is marked with N. In uninfected tumors (Fig. 3.21) several very small necroses could be detected Fig. 3.21A. In the necrotic cells, the nuclei were either very compressed or they had dissolved (Fig. 3.21B). Hardly any neutrophils could be detected in the surrounding of a necrosis in uninfected tumors (Fig. 3.21 and data not shown).

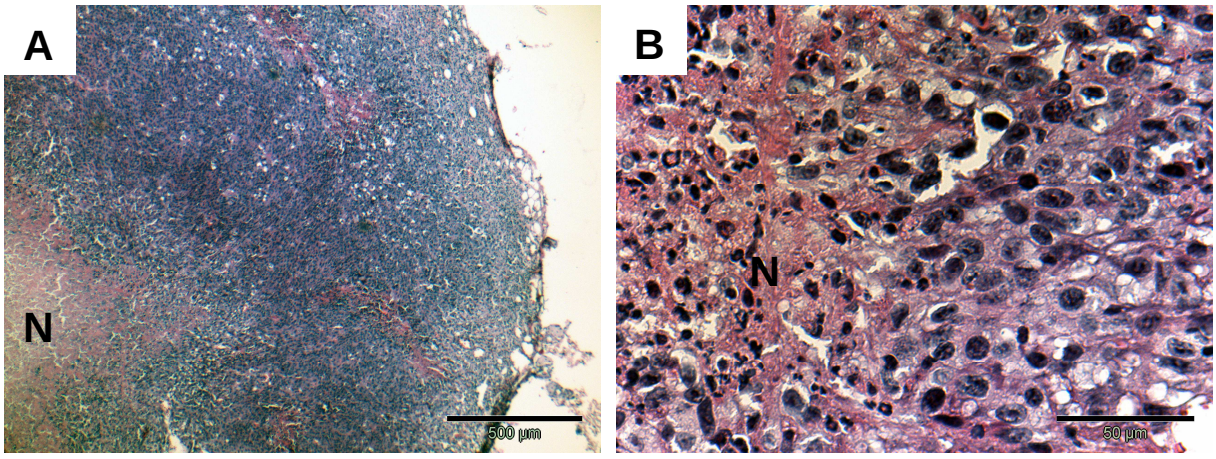



Fig. 3.21: HE staining of uninfected CT26 tumors. Nuclei are stained in violet/blue and the cytoplasm is stained in red/pink. Necrosis is marked with **N**. (**A**) Low magnification overview of an uninfected CT26 tumor. (**B**) Magnified image of a small necrosis. The black bars represent 500 µm in **A** and 50 µm in **B**, respectively.

In contrast, all examined CT26 tumors colonized by bacteria were dominated by one large necrosis, which was surrounded by a border of densely packed cells (compare Fig. 3.22 A, C and E). Even at first glance, the size of necrosis appeared dramatically enlarged. Higher magnifications of these densely packed cells revealed them to be neutrophils, as can be recognized by the multilobulated shape of their nuclei (Fig. 3.22 B, D and F). The basic structures of *S. typhimurium*-infected CT26 tumors Fig. 3.22 A and Fig. 3.22 B, *E. coli*-infected tumors Fig. 3.22 C and Fig. 3.22 D and *S. flexneri*-infected tumors Fig. 3.22 E and Fig. 3.22 F resemble each other and are therefore not bacteria-specific.

Taken together these data absolutely correspond with the data from the cryosections. All tested bacterial strains lead to a tremendous infiltration of host neutrophils, which migrate towards the necrosis and separate the bacteria-containing necrosis from vital tumor tissue.

When depleting the neutrophilic granulocytes by triple injections of 25 µg anti-Gr1 simultaneously to the bacterial infections, the border of densely packed neutrophils had disappeared (Fig. 3.23).

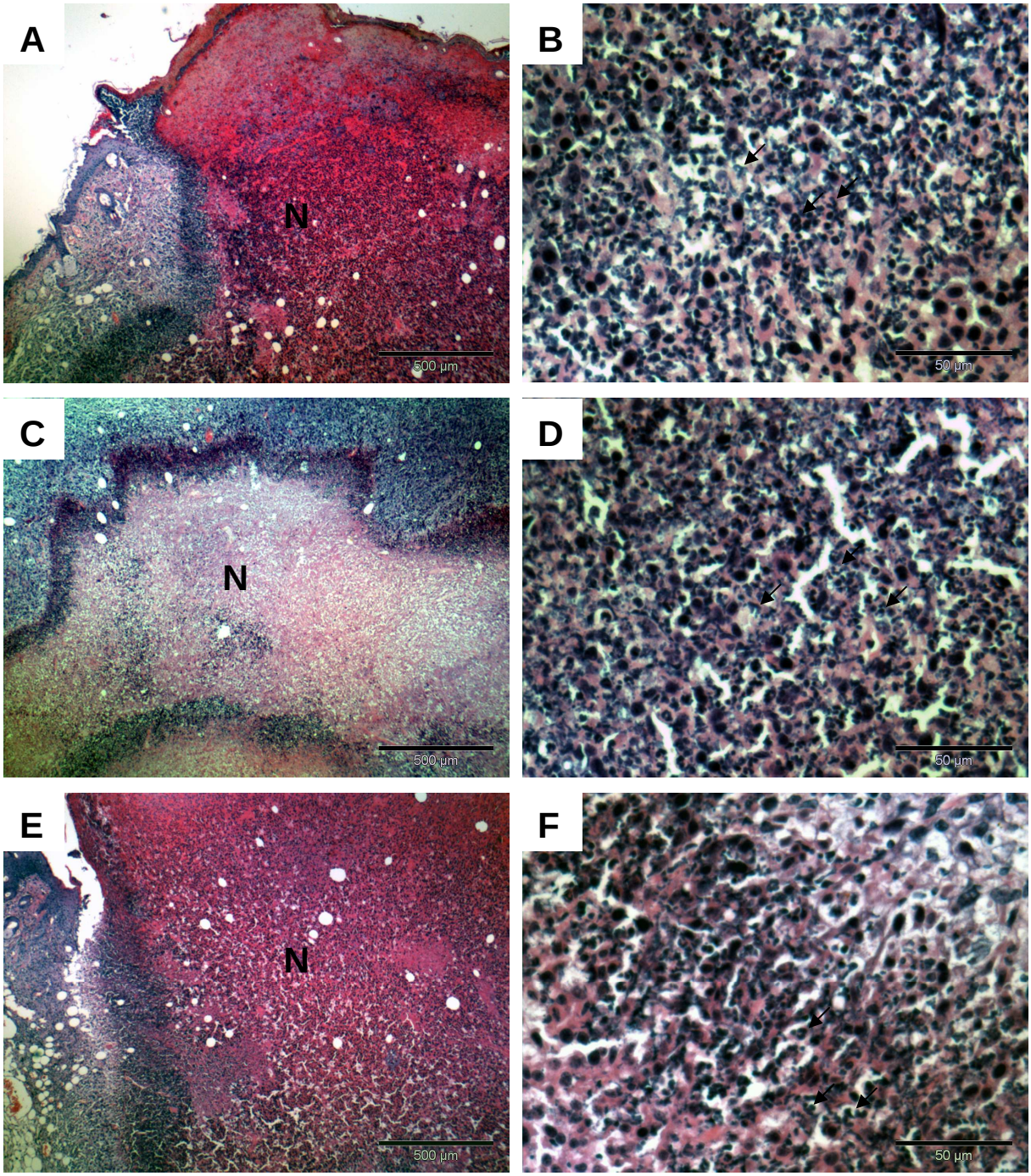



Fig. 3.22: HE staining of infected CT26 tumors. Nuclei are stained in violet/blue and the cytoplasm is stained in red/pink. The necrosis is marked with an **N**. (**A**, **C** and **E**) Low magnification overviews of *S. typhimurium*- (**A**), *E. coli*- (**C**) and *S. flexneri*- (**E**) infected CT26 tumors. (**B**) Magnified images of the neutrophilic border between necrosis and vital tumor tissue of *S. typhimurium*- (**B**), *E. coli*- (**D**) and *S. flexneri*- (**F**) infected CT26 tumors. The black bars represent 500 µm in **A**, **C** and **E** and 50 µm in **B**, **D** and **F**, respectively. The black arrows in **B**, **D** and **F** indicate single neutrophils.

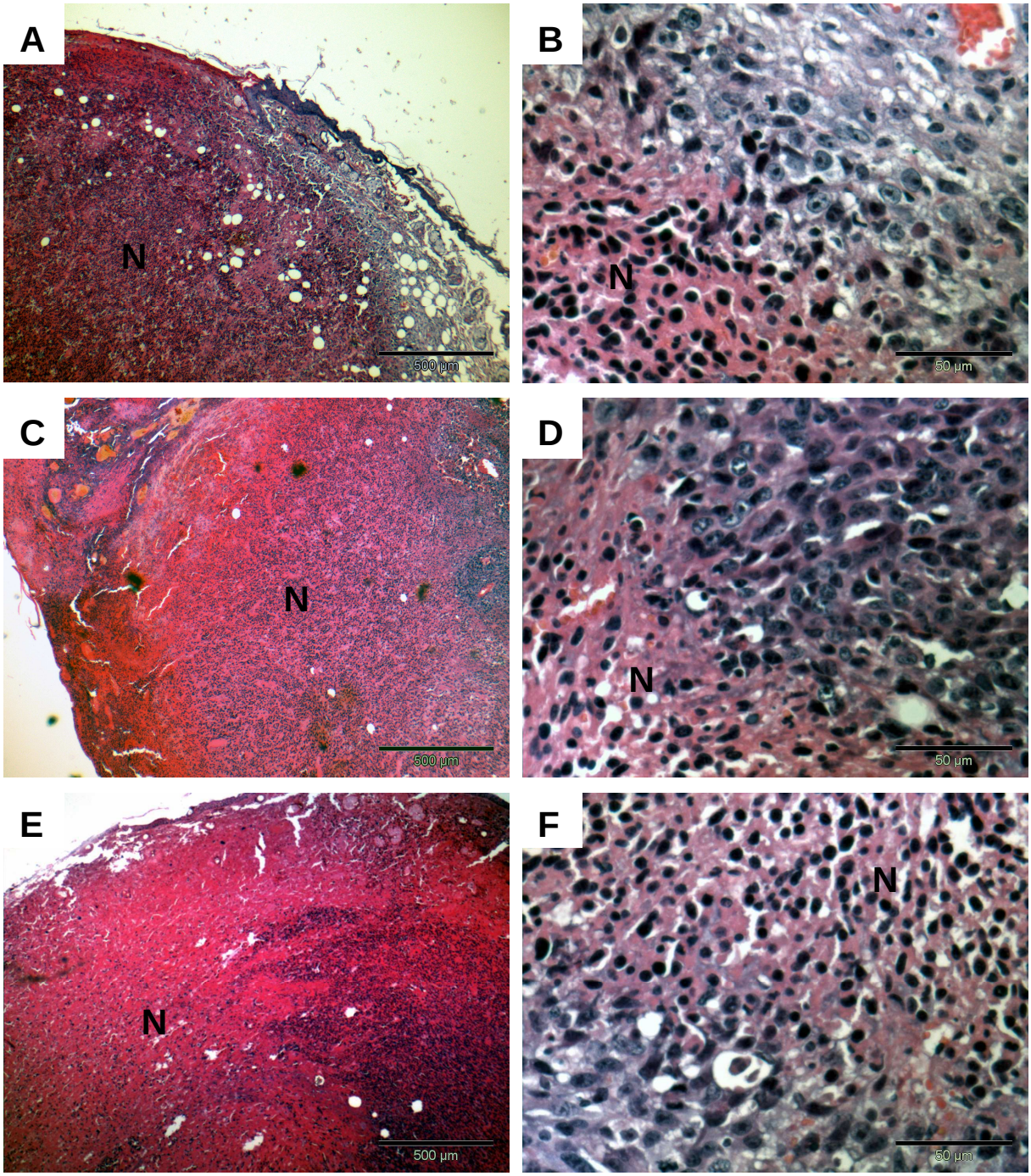



Fig. 3.23: HE staining of infected, neutrophil-depleted CT26 tumors. Nuclei are stained in violet/blue and the cytoplasm is stained in red/pink. The necrosis is marked with **N**. (**A**, **C** and **E**) Low magnification overviews of *S. typhimurium*- (**A**), *E. coli*- (**C**) and *S. flexneri*- (**E**) infected CT26 tumors. (**B**) Magnified images of the border between necrosis and vital tumor tissue of *S. typhimurium*- (**B**), *E. coli*- (**D**) and *S. flexneri*- (**F**) infected, neutrophil-depleted CT26 tumors. The black bars represent 500 µm in **A**, **C** and **E** and 50 µm in **B**, **D** and **F**, respectively.

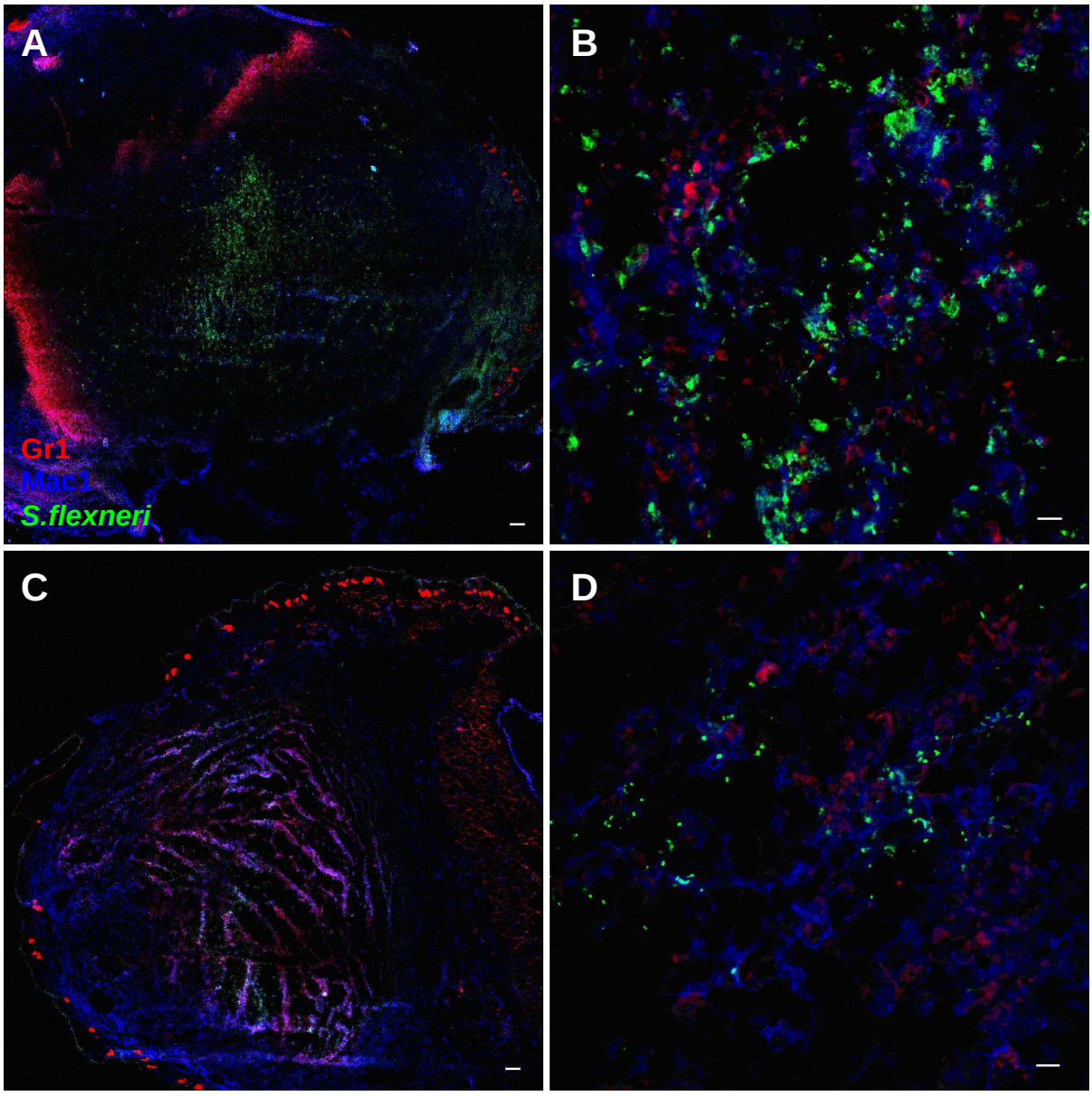



Fig. 3.24: Cryosections of TS/A tumors from *S. flexneri* Δdap-infected mice and from *S. flexneri* Δdap-infected neutrophil-depleted mice were prepared two days p.i. Gr1-positive cells are stained in red, Mac1-positive cells are stained in blue and bacteria are stained in green. (**A**) Low magnification overview of neutrophil dissemination in TS/A tumors 2 days after i.t. infection with *S. flexneri* Δdap. (**B**) Higher magnification of bacteria inside the necrosis. (**C**) Low magnification overview of a neutrophil-depleted, *S. flexneri* Δdap-infected TS/A tumor. (**D**) High magnification of *S. flexneri* in vital tumor tissue of a neutrophil-depleted TS/A tumor. The white bars correspond to 100 µm in **A** and **C** and to 10 µm in **B** and **D**, respectively. Pictures are representative for three tumors from at least two independent experiments.

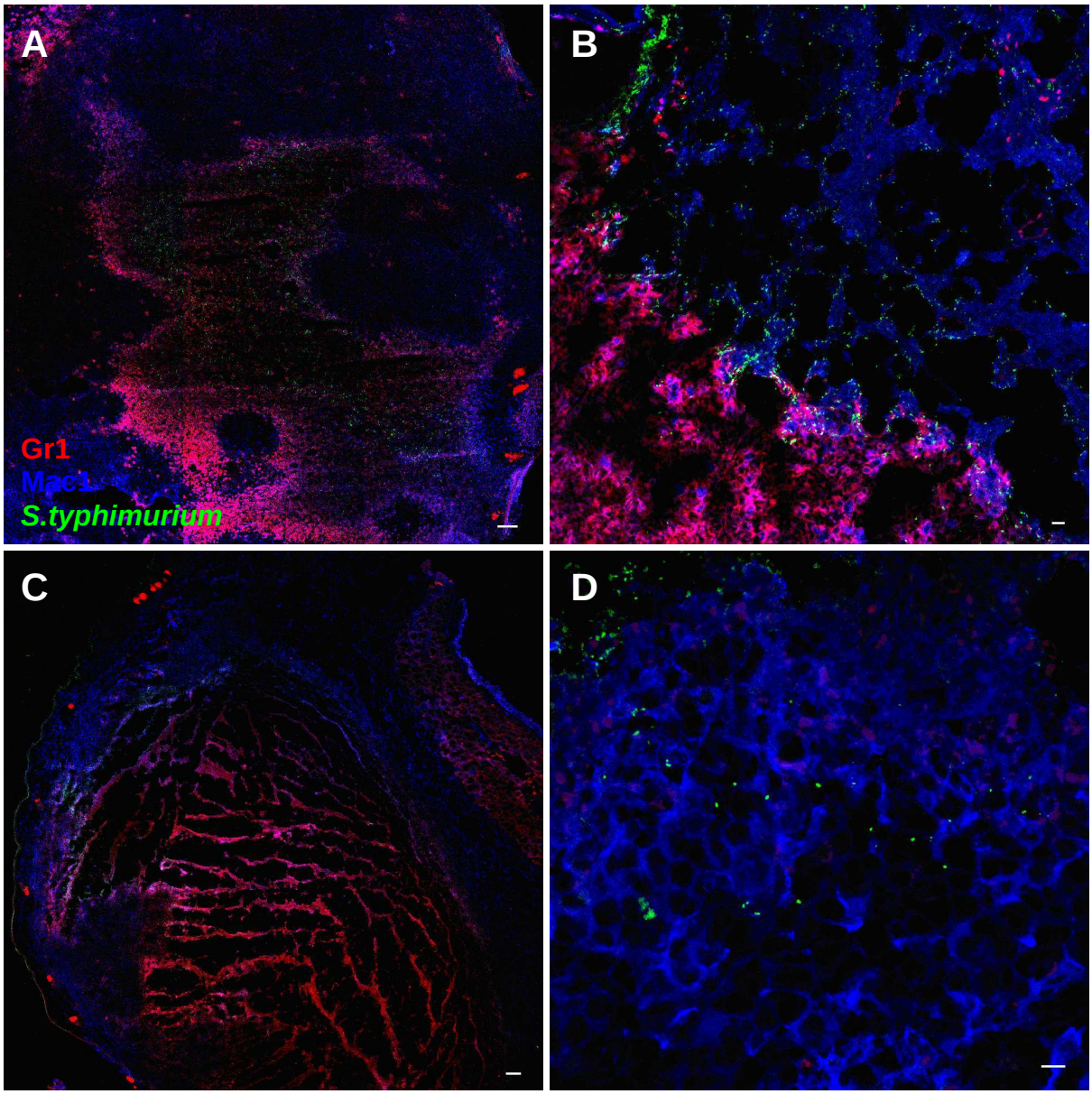



Fig. 3.25: Cryosections of TS/A tumors from *S. typhimurium* SL7207-infected mice and from *S. typhimurium* SL7207-infected neutrophil-depleted mice were prepared 2 days p.i. Gr1-positive cells are stained in red, Mac1-positive cells are stained in blue and bacteria are stained in green. (**A**) Low magnification overview of neutrophil dissemination in TS/A tumors 2 days after i.v. infection with *S. typhimurium* SL7207. (**B**) Higher magnification of the neutrophil border between vital and necrotic tumor tissue. (**C**) Low magnification overview of a neutrophil-depleted, *S. typhimurium* SL7207-infected TS/A tumor. (**D**) High magnification of *S. typhimurium* SL7207 in vital tumor tissue of a neutrophil-depleted TS/A tumor. The white bars correspond to 100 µm in **A** and **C** and to 10 µm in **B** and **D**, respectively. Pictures are representative for three tumors from at least two independent experiments.

Fig. 3.23 A, C and E show overviews of *S. typhimurium*- (Fig. 3.23 A), *E. coli*- (Fig. 3.23 C) and *S. flexneri* (Fig. 3.23 E)-infected, neutrophil-depleted CT26 tumors. All these tumors lack the thick blue border of neutrophils that could be observed in colonized tumors of non-depleted mice (Fig. 3.22). When enlarging the border between necrotic and viable tumor tissue in Gr1-depleted tumors (Fig. 3.23 B, D and F) the first impression obtained from the overviews was confirmed. No more neutrophils were detectable between viable and necrotic tissue. This can be seen by the missing multilobulated nuclei of the cells. Instead, the necrosis, which is more reddish, directly borders the violet stained, vital tumor tissue. As mentioned before, not only the neutrophilic border had disappeared, but also the necrosis had apparently increased.

These results were confirmed with a second subcutaneously injected solid tumor, the adenocarcinoma TS/A. Pictures of stained cryosections of these tumors after infection with *S. typhimurium* SL7207 and with *S. flexneri* Δdap, respectively, with or without neutrophil depletion are shown in Fig. 3.24 and Fig. 3.25.

Regarding gene transfer with *S. flexneri* carrying the reporter plasmid pCMVluc-λ2In-m2A, the depletion of neutrophils did not increase efficiency (data not shown). For optimal gene transfer conditions, the bacteria have to infect viable tumor cells. However, the depletion of neutrophils also leads to an increase in the size of necrosis and thus to an increased number of bacteria that are associated with necrotic, non-viable tumor cells. This is most likely the reason why gene transfer efficiency could not be enhanced.

As the total number of bacteria as well as the spreading of bacteria inside solid tumors could be remarkably improved, other bacteria-mediated tumor therapies, like the delivery of therapeutic proteins via bacterial secretion systems, might be strongly supported by the depletion of neutrophils.

In summary, depletion of neutrophils in the course of bacteria-mediated tumor therapy leads to an enhanced dissemination of bacteria inside solid tumors. Besides a noticeable higher total number of bacteria inside the tumor, an increase of the size of necrosis is observed and most remarkably the migration of bacteria into the vital tumor tissue. The border of host neutrophils that obviously trap the bacteria inside the necrosis had disappeared and the few neutrophils that were left could be circumvented by the bacteria.

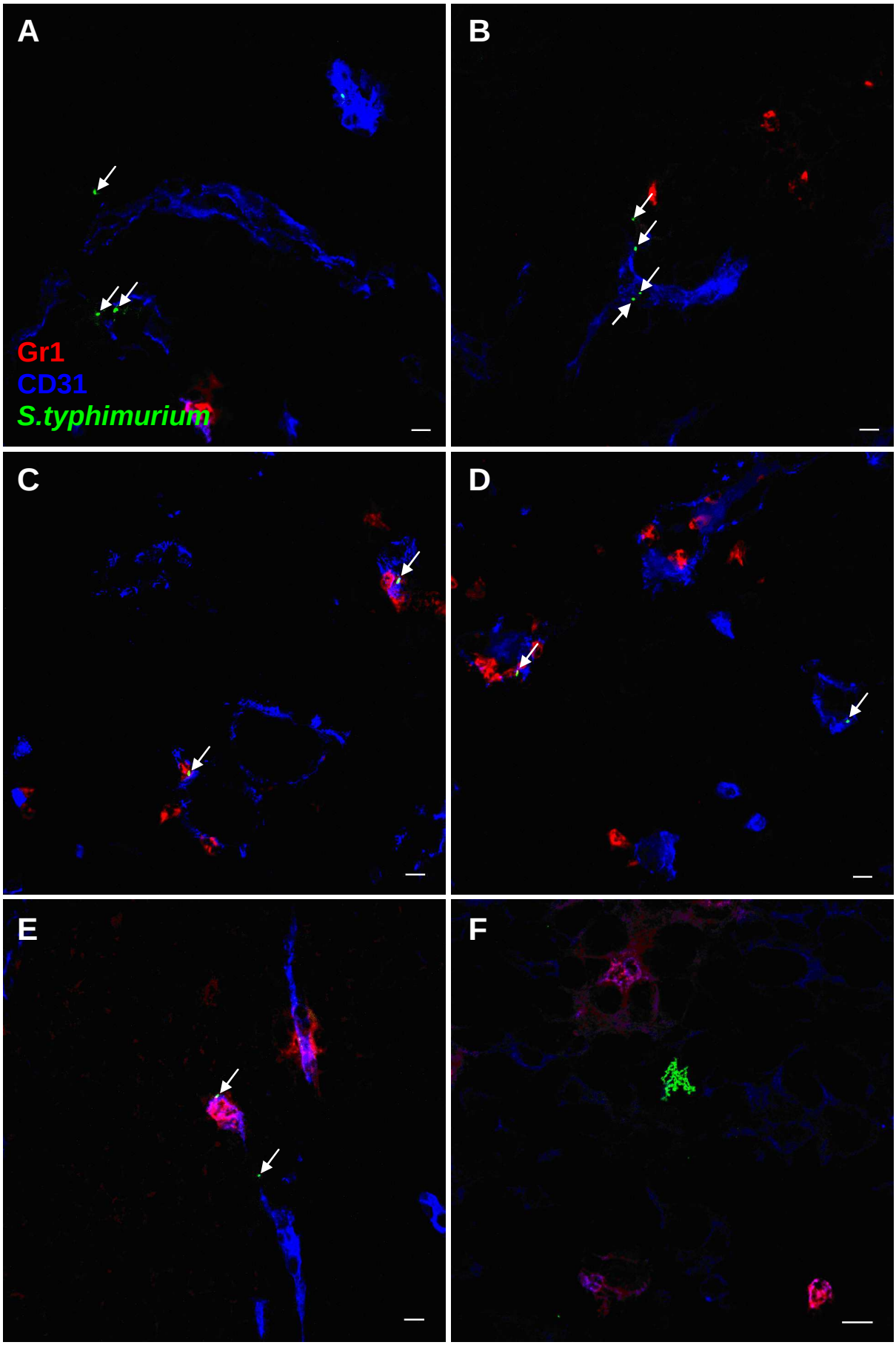
## 3.6 Kinetics of bacterial colonization of solid CT26 tumors

The tremendous influx of neutrophils to the site of infection and the enormous increase of the size of necrosis two days after bacterial infection suggested a closer investigation of the early times of bacterial colonization. Up to now it was claimed that tumor-colonizing bacteria are attracted to hypoxic, especially necrotic areas of solid tumors. The necrosis satisfies the needs of the bacteria in terms of sufficient supply of nutrients, optimal pH and optimal temperature. However, the dramatic increase of the size of necrosis after colonization with facultative anaerobic bacteria suggests that the bacteria themselves induce necrosis after having successfully escaped from the blood into the solid tumor.

In order to investigate, how and when the bacteria start colonizing the tumor and when the infiltration of neutrophils takes place, CT26 tumor-bearing BALB/c mice were intravenously infected with *S. typhimurium* SL7207. At different times after infection, ranging from 30 min to 18 h, tumors were removed and snapfrozen. Cryosections of 10 µm diameters were stained with fluorescently labeled antibodies against *Salmonellae*, neutrophilic granulocytes and CD31, a cluster of differentiation molecule that is normally found on endothelial cells. As the blood vessels are lined with endothelial cells, it should highlight their distribution and their course.

As early as 30 minutes after infection, single bacteria could be found inside blood vessels of CT26 tumors (Fig. 3.26 A and B). The single bacteria are marked with white arrows. Sometimes, it even appears as if a bacterium has escaped a blood vessel (compare the upper left bacterium in Fig. 3.26 A). This situation did not change much over the first six hours after infection (Fig. 3.26 C – E). Most of the bacteria that could be detected were located inside or in close contact to blood vessels and they were isolated. Rarely, tiny clusters or colonies of *Salmonellae* could be detected 6 hours p.i. as depicted in Fig. 3.26 F. Between 6 hours and 15 hours post infection, the tumors were macroscopically becoming dark red, as if blood was flowing in. This phenomenon was analyzed in more detail via hematoxilin/eosin stainings (Fig. 3.28) and is further described below.

Twelve hours post infection these tiny bacterial clusters could be observed more frequently (Fig. 3.26 G and H). They could be found at different places scattered all over the tumor. Often the bacterial colonies were still associated with or in close contact to blood vessels (compare left arrow in Fig. 3.26 G).


A
B
C
D
E
F
Gr1
CD31
S.typhimurium

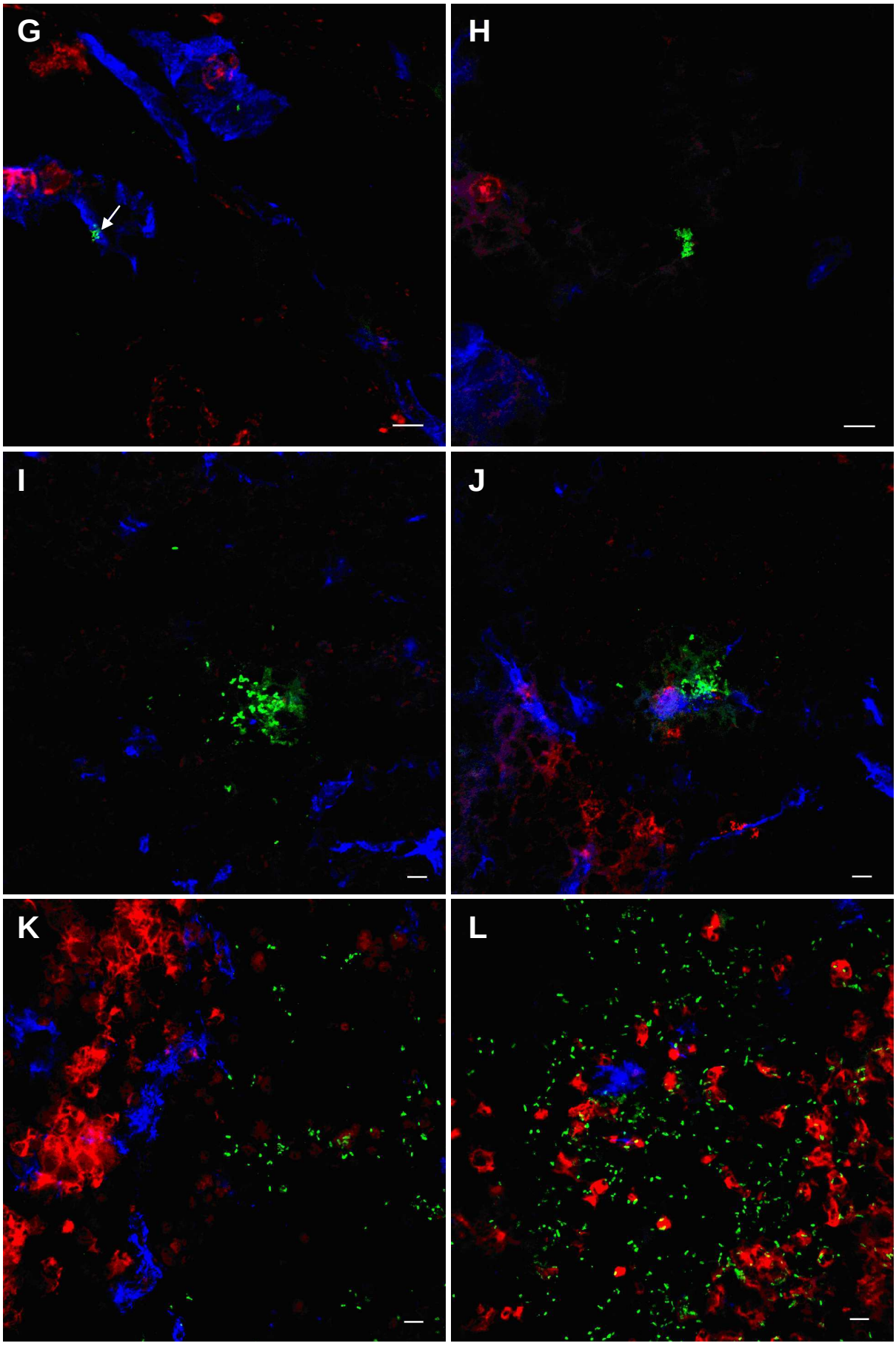

G
H
I
J
K
L

Fig. 3.26: Early times of bacterial colonization of solid CT26 tumors. *S. typhimurium* is stained in green, neutrophils (Gr1) are stained in red and the endothelial cells of blood vessels (CD31) are stained in blue. (**A** and **B**) 30 min post infection. (**C** and **D**) 2 h post infection. (**E** and **F**) 6 h post infection. (**G** and **H**) 12 h post infection. (**I** and **J**) 15 h post infection. (**K** and **L**) 18 h post infection. The white arrows in **A** – **E** indicate single bacteria. The white arrow in **G** indicates a bacterial colony inside a blood vessel. The white bars represent 10 µm.

Fifteen hours post infection the scattered bacterial colonies had become significantly larger (Fig. 3.26 I and J) and the single bacteria were starting to localize at places distant from blood vessels. Eighteen hours post infection (Fig. 3.26 K and L) the situation looked similar to the distribution of bacteria and neutrophils two days p.i.. At this time the bacteria were scattered inside large necrotic areas, which were surrounded by infiltrated neutrophils. Although the bacteria were not as dense as two days post infection and the amount of infiltrated neutrophils was lower, the basic distribution of bacteria and neutrophils was the same. In Fig. 3.26 K and L, the beginning establishment of the border of neutrophils that surrounds the necrotic area and keeps the bacteria from spreading into vital tumor tissue is enlarged. As seen before, *Salmonellae* are found inside the unstained necrotic areas, or in close contact with neutrophils.

Overviews of the infected CT26 tumors revealed that as long as the bacteria had not started to disseminate throughout the tumor neutrophils were scattered all over the solid tumor with a slightly stronger accumulation at the tumor rim. This is visualized in Fig. 3.27 A, which shows neutrophil distribution of blood vessels two hours p.i.. As soon as the bacteria form colonies and start spreading, they seem to induce necrosis, which in turn leads to a stronger influx of neutrophils. Twelve hours p.i. (Fig. 3.27 B) and fifteen hours p.i. (Fig. 3.27 C) the neutrophils migrated towards the infected tissue, which could be seen by an increased accumulation of neutrophils throughout the tumor. At eighteen hours p.i. a small border of neutrophils could be observed that apparently shielded the vital tumor tissue from the quickly multiplying *Salmonellae* (Fig. 3.27 D). The basic pattern of a neutrophil border surrounding the bacteria-colonized necrosis, which had been observed before at two days p.i., could already be seen after 18 h, albeit less pronounced.

As mentioned before, the colonization of solid CT26 tumors with bacteria and the concomitant infiltration of neutrophils to the place of infection that could be observed microscopically, were always associated by a macroscopic reddening of the solid tumor, suggesting a strong influx of blood into the infected tissue.

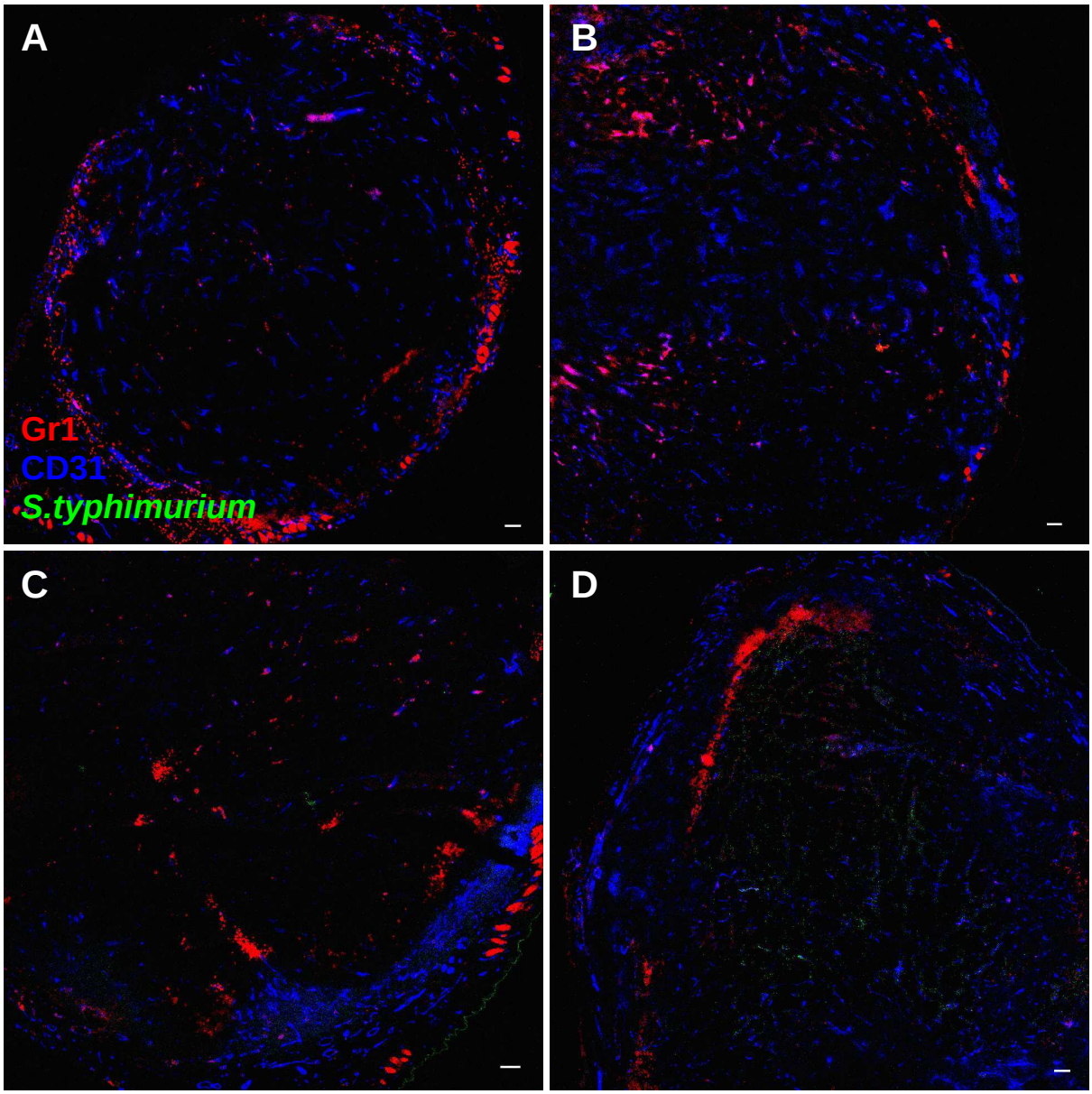



Fig. 3.27: Low magnification overviews of early times during bacterial colonization of solid CT26 tumors. *S. typhimurium* is stained in green, neutrophils (Gr1) are stained in red and the endothelial cells of blood vessels (CD31) are stained in blue. (**A**) 2 h post infection. (**B**) 12 h post infection. (**C**) 15 h post infection. (**D**) 18 h post infection. The white bars represent 100 µm.

To investigate this phenomenon in more detail, 5 µm paraffin sections of infected CT26 tumors that had been infected with *S. typhimurium* twelve hours before were stained with hematoxylin/eosin and analyzed (Fig. 3.27). It became clear that as soon as twelve hours after infection, it is possible to tell where the necrosis is beginning to develop and where the rim of vital tumor tissue will remain. I.e. the bluish violet area directly below the skin, indicated with **V** in Fig. 3.28 will remain vital. Neighboring this area is a huge, more reddish stained area, which will become necrotic (**N**). The border of the area that most likely will remain vital and the area that will become necrotic is enlarged in Fig. 3.28 B. Further enlargement of the

"necrosis-to-be"-area (Fig. 3.28 C and D) revealed the reason for the reddish staining. This area is filled with erythrocytes. Thus, as assumed because of the macroscopic reddening of the solid tumor between six hours and fifteen hours p.i., the tumor is indeed filled up with blood. This is very likely a mechanism via which the neutrophilic granulocytes that have been shown to control intratumoral infections acquire access to the tumor tissue and prevent further spreading of the bacteria. It is conceivable, that blood borne bacteria can also be flushed into the tumor this way.

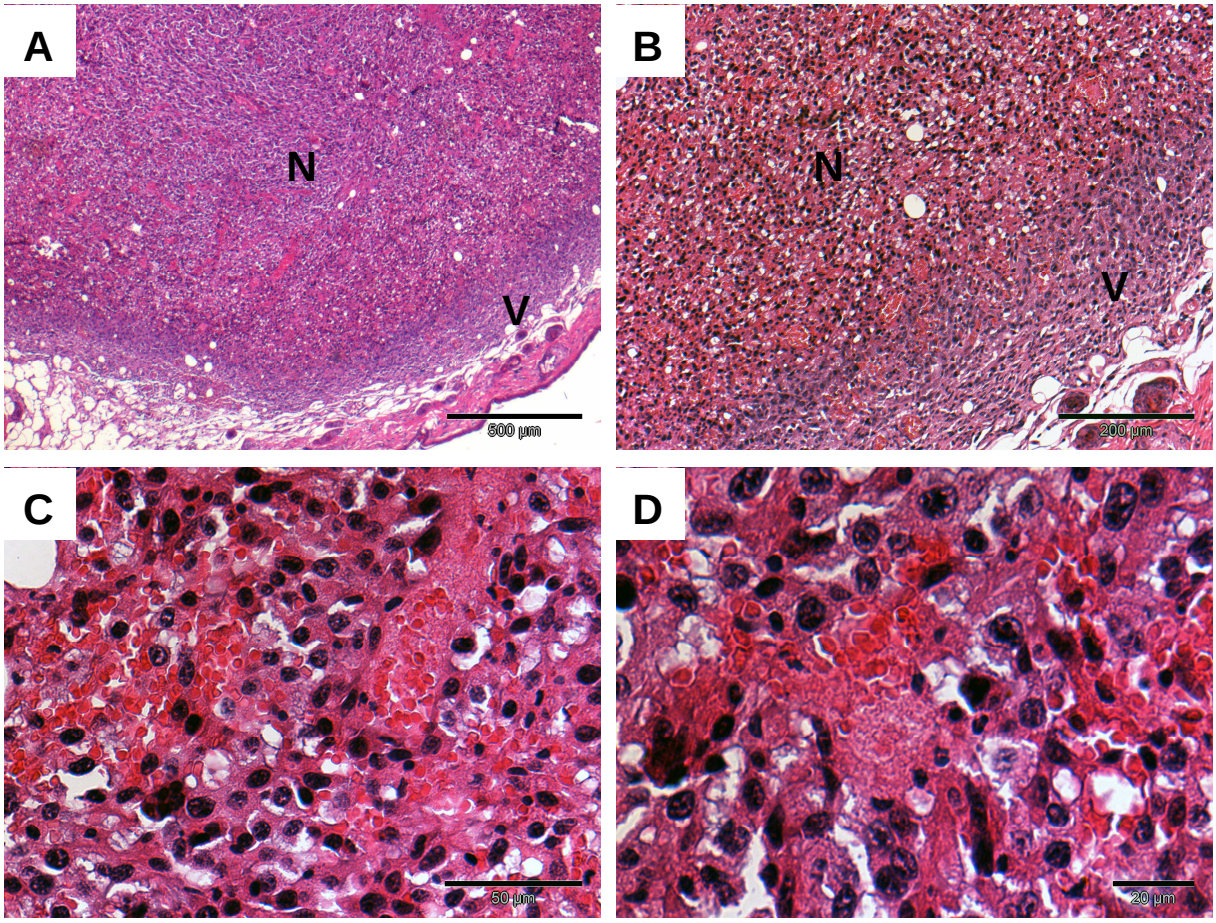



Fig. 3.28: HE stainings of *S. typhimurium* S7207-infected CT26 tumors 12 h p.i.. Nuclei are stained in violet/blue and the cytoplasm is stained in red/pink. The developing necrosis is marked with **N**, the tissue that remains vital is marked with **V**. (**A**) Low magnification overview. (**B**) Higher magnification of the border between the developing necrosis and the tissue that remains vital. (**C** and **D**) High magnification of erythrocytes that have infiltrated the tumor. The black bars represent 500 µm in **A**, 200µm in **B** and 50 µm in **C** and **D**.

## 3.7 *Salmonella typhimurium* SL7207 resides mainly extracellularly inside solid CT26 tumors

An additional important aspect of bacterial settlement inside solid CT26 tumors is the clarification of the question, whether the invasive tumor-colonizing bacteria invade tumor cells, or whether they remain extracellularly.

Therefore, 2µm paraffin sections of *S. typhimurium*-infected CT26 tumors were prepared two days after bacterial infection. The bacteria were stained by a standard silver staining and should appear black. The nuclei were counterstained with Kernechtrot and should appear purple. The cytoplasm should appear pink and the cellular junctions should be obvious as black irregular lines. As demonstrated in Fig. 3.29 A, the bacteria are mainly found in extracellular, unstained white spaces. Fig. 3.29 B shows a part of the viable tumor tissue of the same tumor, in which no *Salmonellae* reside. The insets magnify parts of each picture by 2.5 fold.

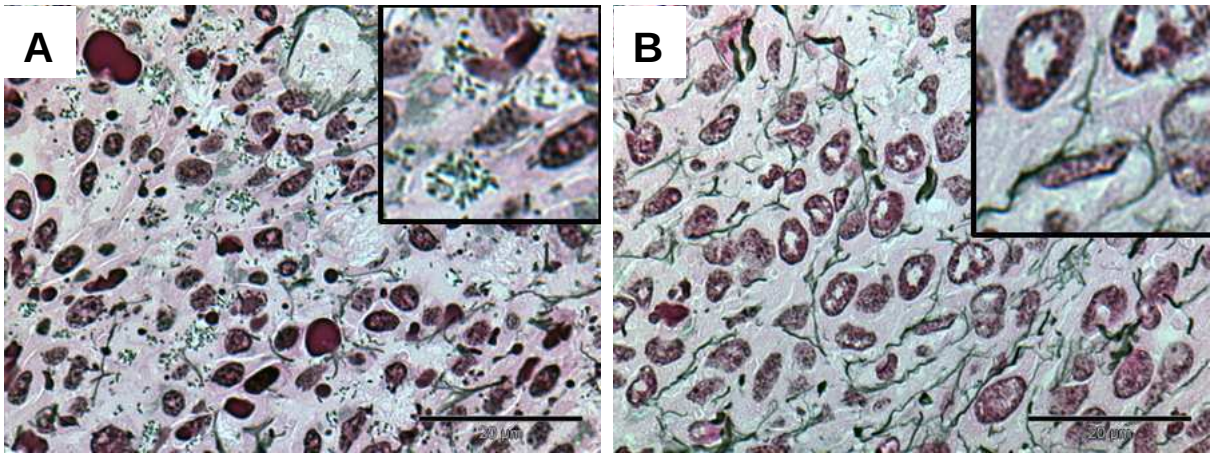



Fig.3.29: Silver staining of histological sections of *S. typhimurium*-infected CT26 tumors two days p.i. Bacteria and cellular junctions appear black. Nuclei are counterstained with Kernechtrot and appear purple. (**A**) Extracellular bacteria at the rim of necrosis. (**B**) Area without bacteria in viable tissue of the same tumor. The insets show 2.5 fold magnifications of a part of each picture. The black bars represent 20 µm.

These results suggested that the majority of the bacteria reside extracellularly. However, an explicit answer to the question whether all bacteria are extracellular or if some bacteria are still able to infect cells and can be found intracellularly could not be established this way.

Therefore, *Salmonella*-infected tumors were analyzed by electron microscopy. CT26 tumors from *S. typhimurium* infected BALB/c mice were removed two days p.i., fixed and prepared

for electron microscope analysis. Fig. 3.30 shows transmission electron micrographs of *Salmonellae* residing in CT26 tumors. Fig. 3.30 A and Fig. 3.30 B clearly show that *S. typhimurium* reside extracellularly. No bacterium could be found to be in direct contact with the cytoplasm. Besides the extracellular occurrence of the bacteria, *S. typhimurium* appears to be encapsulated, as can be seen by the unstained surrounding of the bacteria.

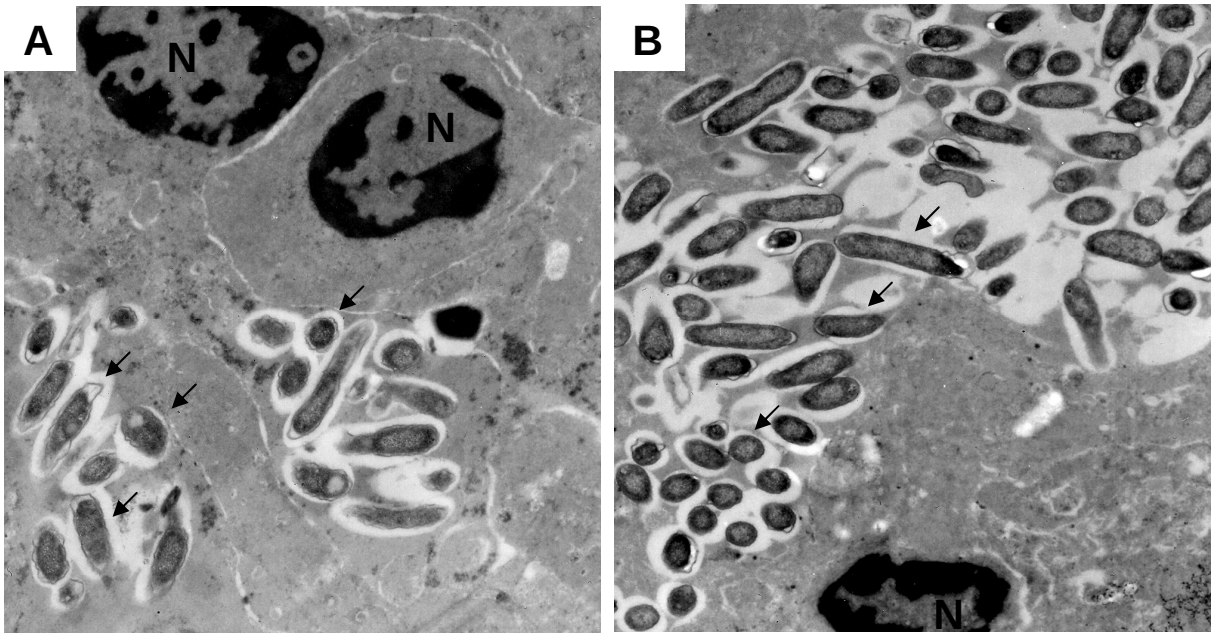



Fig.3.30: *S. typhimurium* reside extracellularly inside CT26 tumors. Transmission electron micrographs of *S. typhimurium* SL7207 infected CT26 tumors two days p.i.. The small dark grey particles are *Salmonellae* are indicated by black arrows. Nuclei are marked with **N**. **A** and **B** highlight different places of *Salmonella* colonization inside CT26 tumors. *Salmonellae* are not in direct contact with the cytoplasm; instead, they stay extracellularly and appear encapsulated.

## 3.8 Bacterial encapsulation and biofilm formation inside solid CT26 tumors

In addition to transmission electron micrographs, freeze fractures of *S. typhimurium*-infected CT26 tumors were analyzed by scanning electron microscopy.

As displayed in Fig. 3.31 *S. typhimurium* 7207 reside in extraordinary structures inside the necrosis of solid CT26 tumors. These sheet-like structures that were always found to surround *Salmonellae* inside the tumors resemble biofilms (Romling and Rohde, 1999; Romling et al., 2000). No cellular structures were visible in the neighborhood of the bacteria.

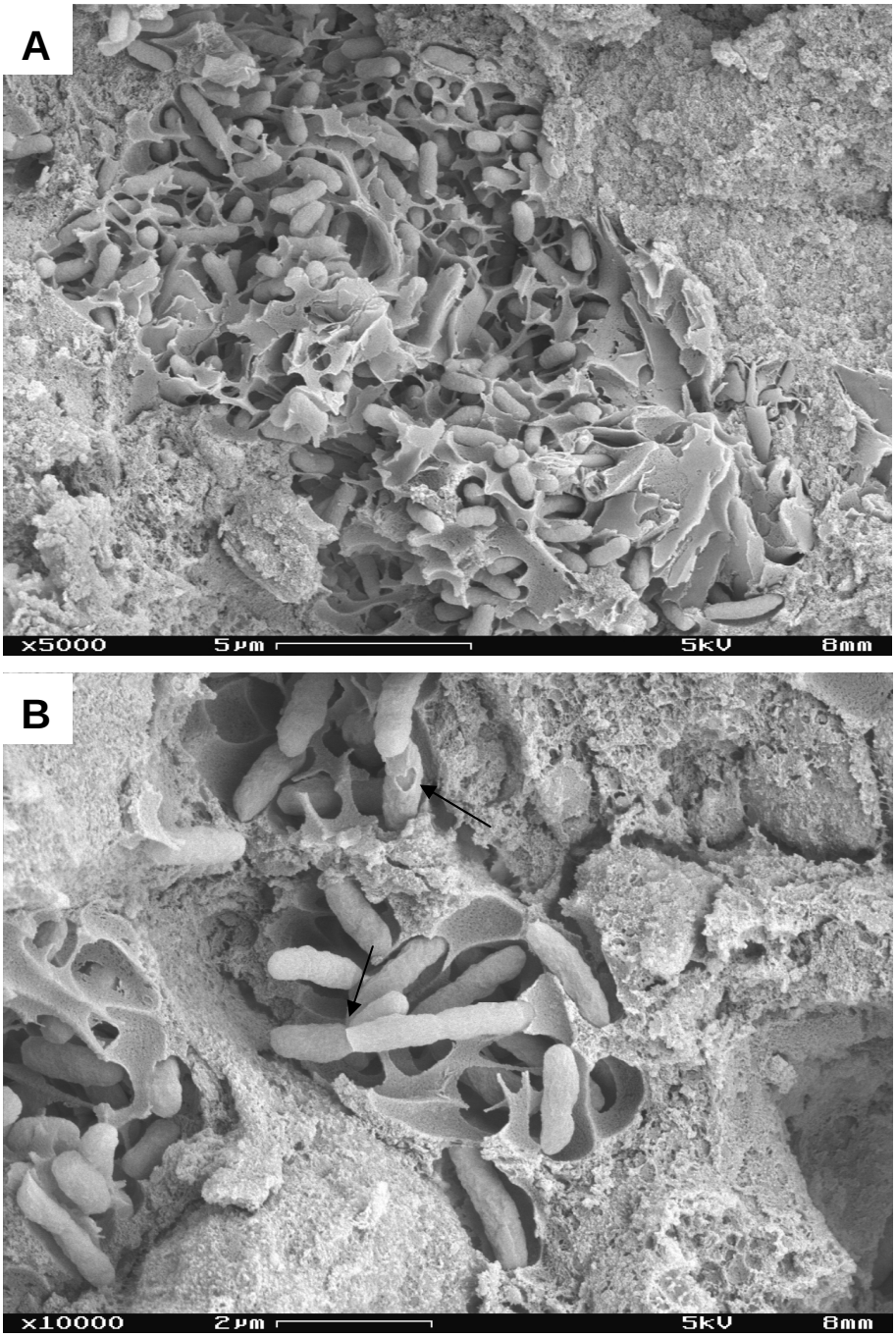



Fig.3.31: *S. typhimurium* are encapsulated and grow in biofilms inside solid CT26 tumors. (**A**) Group of bacteria that is surrounded by extracellular structures that resemble biofilms. No vital cells are detectable. (**B**) Higher magnification of bacteria inside the biofilm. Bacteria are additionally encapsulated. The upper black arrow indicates a capsule that had been damaged during fracture; the lower black arrow indicates a bacterium that migrates out of a tube-like capsule. The white bars represent 5 µm (**A**) and 2 µm (**B**), respectively.

Besides the formation of biofilms by *S. typhimurium* inside CT26 tumors, the bacteria were found to be encapsulated. In Fig. 3.31 B these capsules that surround the *Salmonellae* are highlighted by black arrows. The upper arrow indicates a capsule that was obviously damaged during the fracturing procedure of the tumor. The lower arrow marks a single bacterium that appears to outgrow a tube-like capsule.

In summary, *Salmonellae* that were found to inhabit the necrosis of solid CT26 tumors were surrounded by capsules and they additionally form biofilms. The encapsulation and the biofilm formation might be a protection mechanism of the bacteria, to avoid contact and phagocytosis by host immune cells like neutrophilic granulocytes.

In fact, when analyzing freeze fractures of *S. typhimurium*-infected, neutrophil-depleted CT26 tumors via electron microscopy, biofilm formation was retarded and most of the *Salmonellae* were not encapsulated. Fig. 3.32 shows *S. typhimurium* in neutrophil-depleted solid CT26 tumors. Contrary to the non-depleted tumors, the bacteria were not associated with the sheet-like structures of a biofilm as in Fig. 3.31. One single ripped capsule was detectable in Fig. 3.32 A, as highlighted by the black arrow. Irrespective of the capsule in Fig. 3.32 A, hardly any further encapsulated bacteria could be detected. In Fig. 3.32 B it is clearly visible that the bacteria were not encapsulated, as the ruffled structure of the bacterial membrane is visible. This differs remarkably from the outer structures of the capsules in Fig. 3.31. Despite of this phenomenon, the bacteria remain extracellularly. Obviously, additional mechanisms must exist that keep the bacteria in this state.

Taken together, these data indicate that *S. typhimurium* are induced to form biofilms and capsules upon contact to neutrophilic granulocytes. Both mechanisms seem to be a protection mechanism of the bacteria, to prevent phagocytosis and/or killing by host neutrophils. In addition, encapsulation and biofilm formation might partly explain, why *S. typhimurium* is not found intracellularly.

In contrast to *S. typhimurium* SL7207, the laboratory strain *E. coli* TOP10 did not show any biofilm formation or encapsulation inside solid CT26 tumors. As can be seen in Fig. 3.33 rod-shaped *E. coli* are located between damaged, necrotic tumor tissue without extraordinary structures like biofilms or capsules. The appearance of *E. coli* TOP10 in Fig. 3.33 B resembles the appearance of *S. typhimurium* in neutrophil-depleted tumors.

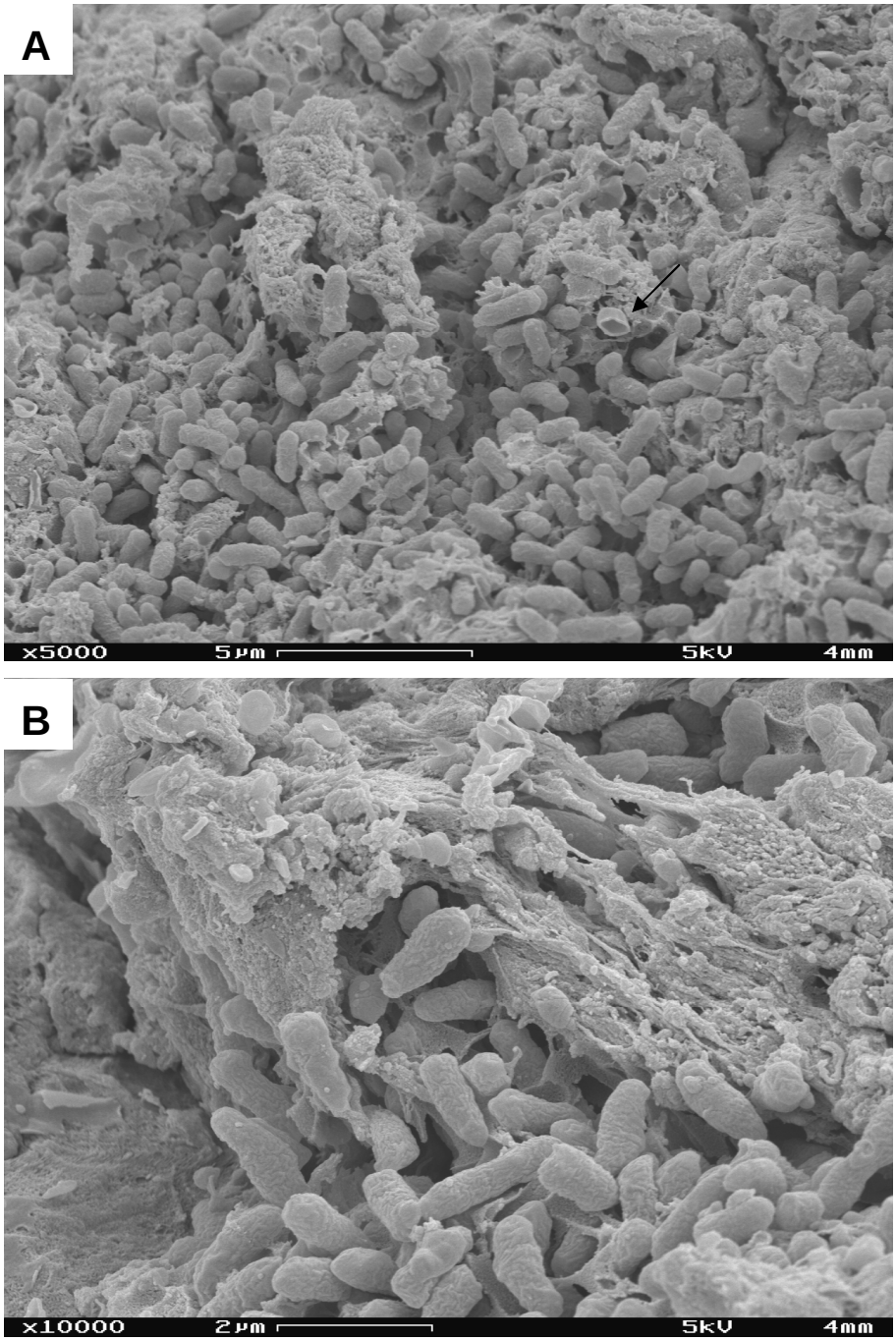



Fig.3.32: Biofilm formation and encapsulation by *S. typhimurium* is retarded in neutrophil-depleted CT26 tumors. (**A**) *Salmonellae* inside neutrophil-depleted CT26 tumors. Bacteria are not surrounded by the sheet-like structures of the biofilm. The black arrow indicates one single ripped bacterial capsule. (**B**) Higher magnification of the bacteria. Bacteria are not encapsulated. Structures of the bacterial membranes are visible. The white bars represent 5 µm (**A**) and 2 µm (**B**), respectively.

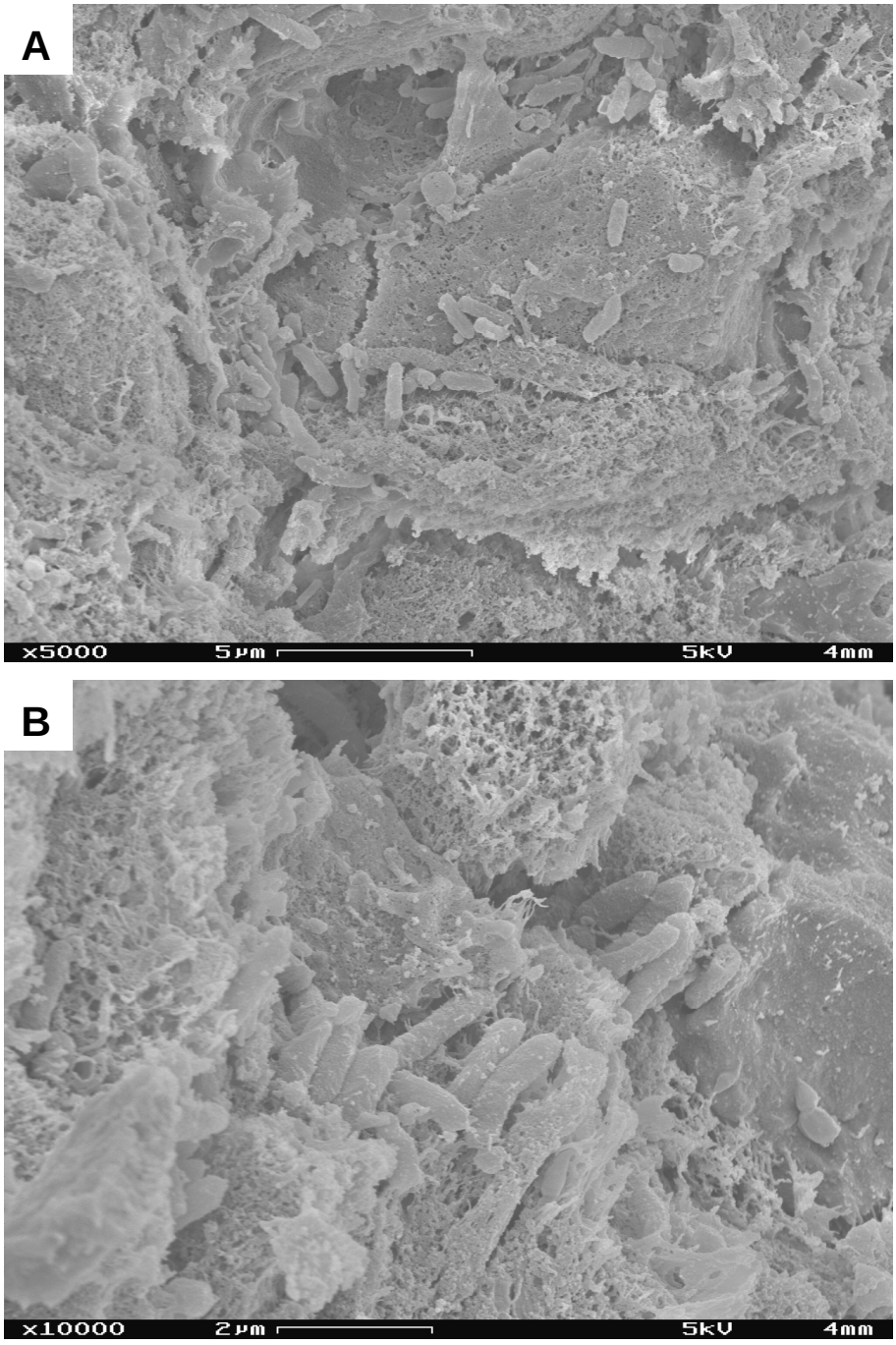



Fig.3.33: No biofilm formation and encapsulation by *E. coli* TOP10 inside CT26 tumors. (**A**) *E. coli* inside the necrosis of CT26 tumors. Bacteria are not surrounded by the sheet-like structures of a biofilm. (**B**) Higher magnification of the bacteria. Bacteria are not encapsulated. The white bars represent 5 µm (**A**) and 2 µm (**B**), respectively.

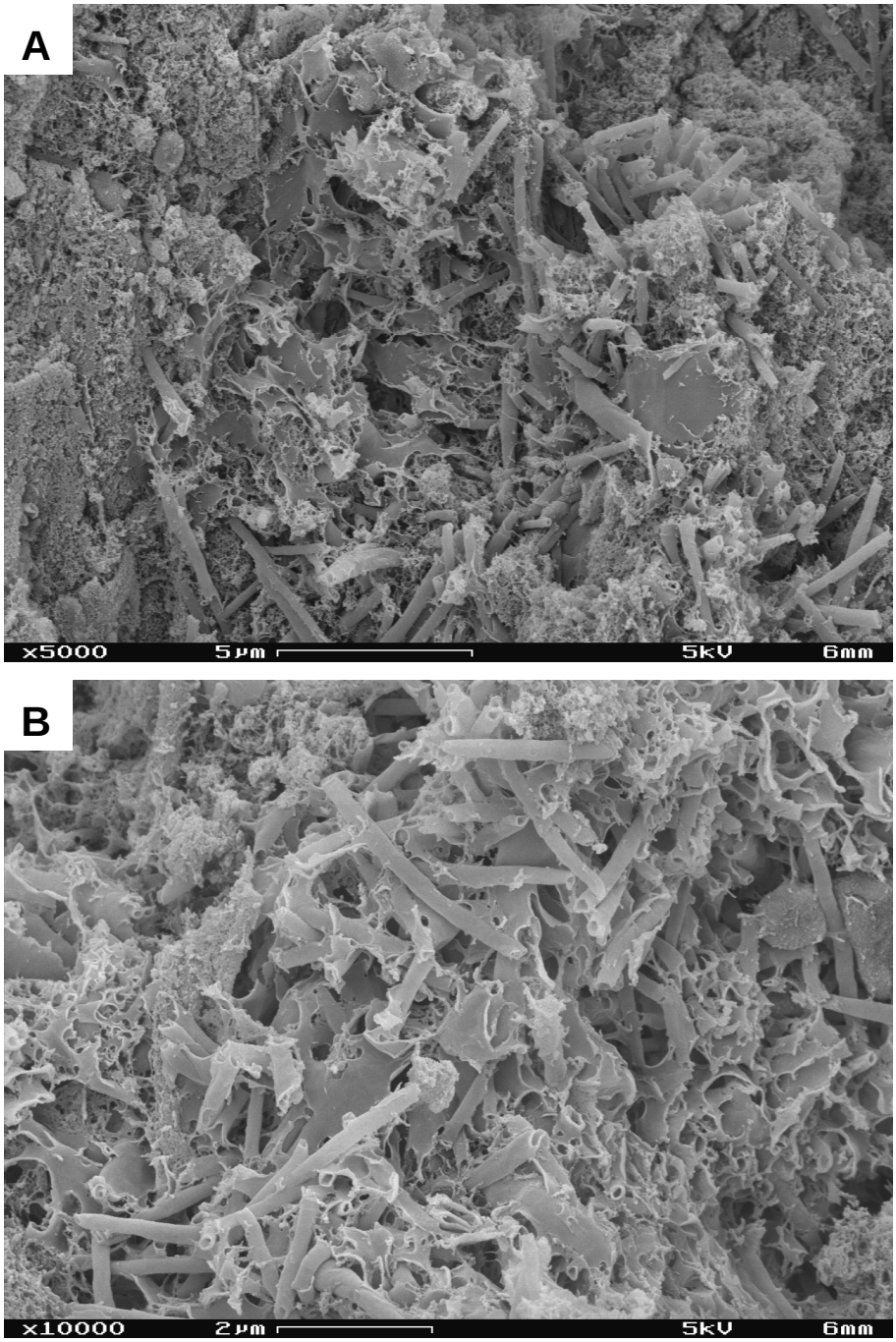



Fig.3.34: Biofilm formation and encapsulation by *S. flexneri* Δdap in neutrophil-depleted CT26 tumors. (**A**) *Shigellae* inside neutrophil-depleted CT26 tumors. Bacteria form biofilms and are encapsulated by tube-shaped capsules (**B**) Higher magnification of the bacteria inside tube-shaped capsules. The white bars represent 5 µm (**A**) and 2 µm (**B**), respectively.

These results correlate well with published data from Romling and Rohde (1999) and Romling et al. (2000), who reported multicellular behavior and biofilm formation in several *S. typhimurium* and *E. coli* species. According to their studies, most *E. coli* K12 derivatives do not show multicellular behavior. As the laboratory strain *E. coli* TOP10 is a K12 derivative, its inability to form biofilms would be expected.

The third bacterial strain that was analyzed via electron microscopy, *S. flexneri* Δdap, again behaved differently inside solid CT26 tumors. Similar to *S. typhimurium* SL7207, *S. flexneri* Δdap did exhibit biofilm formation and encapsulation in non-depleted CT26 tumors (data not shown). Interestingly, even after successful depletion of neutrophils, neither biofilm formation nor encapsulation of *Shigellae* seemed to be impaired, as displayed in Fig. 3.34. In addition, clear differences between biofilm and capsules of *Shigellae* and *Salmonellae* became apparent.

The capsules of *S. flexneri* Δdap (Fig. 3.34 A and B) appear thinner than *S. typhimurium*'s (Fig. 3.31 B) and resemble thin tubes. Such tubes appear to be open on both sides (Fig. 3.34 B). In case of *Salmonella*, most of the bacteria were individually encapsulated, as only few tube-like structures could be observed. In contrast, *Shigellae* appear not to be encapsulated individually (compare Fig. 3.34 B). In addition, the sheet-like structure of *Shigella flexneri*'s biofilm differs from the structure of *S. typhimurium*'s biofilm.

Since biofilm formation and encapsulation of *S. flexneri* Δdap were not impaired in neutrophil-depleted tumors, the mechanisms that are responsible for the formation of biofilms and capsules by *S. flexneri* must differ from the mechanisms that cause biofilm and capsule formation by *S. typhimurium*. Nevertheless, the formation of biofilm and capsules by the two bacterial strains was surprising. It might even partly explain the failure of gene transfer. On the other hand it opens a wide range of possibilities to future tumor therapies.

# 4 Discussion

Facultative and obligate anaerobic bacteria as means for tumor therapy have been exploited experimentally for several decades. Different kinds of bacteria-mediated tumor-therapies have been tested so far and some of them showed very promising results. However, little is known about the mechanisms that are involved in bacterial colonization of solid tumors and on bacteria-host interactions inside the tumor.

When dealing with therapeutic applications, such as bacteria-mediated tumor therapy, it is essential to know every detail possible in order to optimize the treatment or to prevent unexpected side effects.

The present work started by verifying functional gene transfer of a reporter gene into solid CT26 tumors with *S. flexneri* Δdap as carrier. Prompted by fluctuating *in vivo* results, investigations turned towards characterization of interactions of *S. flexneri* Δdap with the mammalian host. These studies were extended to two additional carriers *S. typhimurium* SL7207 and *E. coli* TOP10.

Electron microscopy unveiled an unexpected encapsulation and the formation of biofilms by the two invasive strains *S. typhimurium* SL7207 and *S. flexneri* Δdap inside CT26 tumors. Remarkably, the multicellular behavior was not observed for the noninvasive strain *E. coli* TOP10. Both, the encapsulation and the biofilm formation of *S. flexneri* Δdap might explain the fluctuating results of *in vivo* gene transfer experiments.

The second unexpected finding was the enrichment of neutrophils at the place of infection. These cells apparently were responsible for the containment of bacteria to the necrotic parts of the tumor. Since such infiltrating neutrophils might have adverse effects on bacterial dissemination inside the tumors, the depletion of neutrophils was investigated. As intended, the depletion enhanced the dissemination of bacteria. In addition, an increase in necrosis and spreading of the bacteria into vital tumor tissue could be achieved.

The characterization of bacteria-host interactions further included the kinetics of bacterial settlement inside solid CT26 tumors. *S. typhimurium* SL7207 were taken as a representative tumor-targeting bacterium and were found already short time after administration in the tumor associated with blood vessels. Between 6 h and 15 h a tremendous influx of blood was observed, which was accompanied by necrosis formation, bacterial growth and the above described infiltration of the tumor by neutrophils. After about 24 hours the final scenario for tumors colonized by bacteria was established.

## 4.1 Early events in bacterial tumor colonization

**Bacterial escape into the solid tumor**

As early as 30 min to 2 h after systemic infection, the first *S. typhimurium* SL7207 could be detected histologically in blood vessels of CT26 tumors. In rare cases they were found even inside the tumor tissue but still in close proximity to blood vessels. These data are in agreement with plating experiments of tumors and blood samples from intravenously infected, tumor-bearing BALB/c mice (Leschner, S., personal communication). Elimination of *S. typhimurium* from the blood takes place between 2-6 hours after infection and is accompanied by a simultaneous increase of bacterial numbers in the tumor. These results are in contrast to a publication by Forbes et al. (2003), who reported the elimination of *S. typhimurium* from the blood of MCaIV tumor-bearing SCID-mice after 24 h. The discrepancy might be explained by the different mouse strains employed. SCID-mice are severe combined immune deficiency mice that cannot produce functional T or B cells and therefore do not contain natural antibodies. Such antibodies have been shown to be essential for spleen-specific clearance of pathogens from the circulation (MacPherson et al., 2000).

The exact mechanism of how the bacteria escape into the tumor tissue is not yet clear. Basically, two mechanisms are conceivable: the bacteria are transported into the tumor passively or they actively escape from the blood into the tumor tissue.

A very plausible mechanism is a passive infiltration of bacteria into the tumor. Most tumor vessels differ from normal blood vessels, as they are structurally irregular and leaky, with irregular diameters and abnormal branching patterns (Less et al., 1991; Less et al., 1997). In 2000, Hashizume et al. studied the leakiness of blood vessels in MCaIV mouse mammary tumors and concluded that some tumor vessels have a defective cellular lining that is composed of disorganized, loosely connected, branched or overlapping endothelial cells. The openings between those disorganized cells do not only contribute to blood vessel leakiness in the tumor, but they also permit the access of macromolecules to the tumor. The size of these openings was shown to range between 200 and 2000 nm, depending on the tumor (Hashizume et al., 2000). These conclusions were based on experiments using liposomes, which are relatively large inert particles that can penetrate tumors through fenestrated endothelium (Hashizume et al., 2000). Similarly, Cheong et al. (Cheong et al., 2006) successfully used liposome-encapsulated therapeutics in CT26-bearing BALB/c mice. By extrapolation, it can be assumed that bacteria as well are able to access tumors via such openings.

An example of an active transportation is a receptor-mediated mechanism, in which the bacteria would have to bind to specific receptors found on endothelial cells that line the blood vessels of solid tumors, thereby inducing their own uptake into the solid tumors. Forbes et al.

(Forbes et al., 2003) investigated the escape of bacteria from the blood vessels into tumor tissue. *Salmonellae* were observed to only adhere to the walls of blood vessels with very low flow rates. It can be imagined that the bacteria rested in dead end blood vessels or in niches of irregularly shaped blood vessels with low blood flow rates. Thus, even weak adherence to endothelia would facilitate bacterial escape into the tumor via the large openings in the vessel walls.

In a second example of active transportation bacteria could be specifically attracted by tumors. In 2006 Kasinskas and Forbes observed that *S. typhimurium* are specifically chemotaxed by tumor cells. They hypothesized the production of specific compounds by quiescent cancer cells inside solid tumors. These compounds – although still unknown – could explain the targeting of several facultative anaerobic bacteria to solid tumors (Kasinskas and Forbes, 2006).

Both active mechanisms would predict that relatively high numbers of bacteria simultaneously bind to endothelial cells or infiltrate the solid tumor. This was actually not observed in the present work. In contrast, only single *Salmonella* could be found inside tumor blood vessels 30 min to 6 h after infection and even fewer were found to have escaped into the tumor tissue. During the time frame between 6 h and 12 h post infection, rare micro-colonies could be detected, from which the bacteria started to spread into the tumor tissue. These observations cannot be reconciled with the proposed active mechanisms.

**Influx of blood**

Shortly after the escape of single bacteria into the tumor tissue, the invaded bacteria were shown to proliferate and start disseminating throughout the tumor. This was accompanied by a strong influx of blood into the infected tissue. It is assumed here that the bacteria inside the tumor induce the expression and secretion of cytokines or chemokines by the tumor cells or by macrophages or neutrophilic granulocytes that were found scattered over the non-infected, solid tumors. A prime candidate of secreted factors is tumor necrosis factor α (TNF-α). TNF-α has often been shown to be secreted quickly after infection. In addition, TNF-α is known to act as a vasoactive agent that modifies the actin cytoskeleton and increases the permeability of the endothelial lining of blood vessels (Wojciak-Stothard and Ridley, 2002). This may well support in the influx of blood observed 6 h to 15 h post infection, macroscopically and microscopically.

**Attraction and infiltration of neutrophils**

Concomitant to the influx of blood, neutrophils are migrating into the colonized tumor. Very likely, such neutrophils are flushed into the tumor, together with the inflowing blood and the erythrocytes, as the number of neutrophils inside the tumors slowly increases between 6 h and 18 h post infection. In addition, specific immigration of neutrophils will be induced by bacterial products like N-formylated peptides or the secreted chemokines and cytokines mentioned above. Besides TNF-α, secreted neutrophil attracting factors could include epithelial-derived neutrophil attractant (ENA; CXCL5), growth-related oncogene α (gro-α; CXCL1); monocyte chemoattractant chemokine 1 (MCP-1; CCL2), interferon gamma inducible protein 10 (IP-10; CXCL10) and monokine induced by interferon gamma (MIG; CXCL9). Such chemokines should be amongst the first chemoattractants to be tested in this context in tumor cells and tumor infiltrating phagocytes.

Once inside the tumor, the neutrophils start to border the infected, partly necrotic tumor tissue and are most likely responsible for the local containment of the bacteria.

**Establishment of necrosis and development of a viable tumor rim**

Whenever analyzing tumors colonized by bacteria, the microorganisms are mainly restricted to necrosis, while an outer rim of viable tumor cells invariably persists (Minton et al., 2001; Dang et al., 2001, Forbes et al., 2003). The large, bacteria-colonized necrosis is most probably a result of the bacteria-induced influx of blood. Besides several types of cells, blood contains proteins like clotting factors, complement etc.. Inside the tumor, enzymatic cascades are activated and the released blood begins to clot. Tissue in contact with the clotted blood becomes necrotic.

A second cause of the emerging necrosis could be the disintegration of blood vessels in the hemorrhagic region and the resulting collapse of blood flow. As a result, the constant supply of the tumor cells with nutrients and oxygen is stopped. Thus, the tumor cells in the center of the tumor are “starved and suffocated to death”.

The observed viable rim consists of the remaining tumor tissue that was not in contact with the inflowing blood and is still supplied with nutrients and oxygen. Interestingly, the same phenomenon of a viable tumor rim was reported for solid tumors that were treated with vascular-disrupting agents (VDAs) (Tozer et al., 1999; reviewed in Tozer et al., 2005a). Tumor center and periphery can display different interstitial blood pressure and vascular architecture. Thus, tumor periphery might be less sensitive to vascular shutdown than the center. As the interstitial blood pressure rises strongly from tumor periphery to tumor center,

an increase in vascular permeability might be catastrophic in the center, while it is tolerated in the periphery (reviewed in Tozer et al. 2005a).

A second factor that might play a role is the complex vascular plexus at the tumor periphery compared with a much lower vascular density in the center. In case of extensive vascular damage, residual blood flow is likely to persist in the tumor periphery, but less likely in the tumor center. According to Tozer et al., this can indeed be observed by microscopic techniques (Tozer et al., 2005b).

The mechanisms that are suggested to play a role for the tumor physiology after treatment with VDAs, very likely are the same that lead to necrosis and a viable rim in bacterial tumor therapies. Although the increase in vessel permeability is induced differently in both therapies, the observations and the outcome are very similar.

Tozer and his colleagues also found an influx of neutrophilic granulocytes into the solid tumor. Although in their case the increase in blood vessel permeability was initially due to VDAs, the dying, necrotic cells might have subsequently released neutrophil-attracting factors that lead to an immigration of neutrophils. In case of tumor colonizing bacteria neutrophil-attracting factors from necrotic cells might act in synergy with the chemoattractants induced by the microorganisms.

## 4.2 Containment of tumor colonizing bacteria by host neutrophils

The strong influx of neutrophils into the tumor after bacterial settlement has severe consequences. By limiting the dissemination of the microorganisms, this will most likely lead to restricted efficiency of tumor therapies in terms of (I) equal distribution of cytotoxins or other therapeutics that the bacteria transport into the tumor, and (II) direct contact of the bacteria with vital tumor cells and tumor cell invasion. The latter would be absolutely necessary for gene therapies, e.g. bacteria-mediated gene transfer (Grillot-Courvalin et al., 2002; Lee et al., 2004) or direct injection of therapeutic proteins or RNA into tumor cells. Thus, one of the most urgent issues that have to be addressed is the improvement of bacterial dissemination inside solid tumors.

Inside the tumor, neutrophils probably function as a “border” of leukocytes that intent to control the infection. Thereby, they inhibit bacterial dissemination into other parts of the body, but also into the vital tumor tissue. This role of neutrophils as the first line of defense during bacterial infections can be life-saving in common, unintended infections, but it is most likely counteracting when it comes to bacteria-mediated anti-tumor therapy. Indeed, after depletion of neutrophils from CT26- and TS/A-tumor bearing BALB/c mice all bacteria tested

were able to improve their dissemination throughout the tumor. This was accompanied by higher total numbers of bacteria inside the tumor and an increase in necrosis. Most importantly, bacteria could now be detected in vital tumor tissue.

However, the depletion of neutrophils in tumor patients might appear as a “cruel intention”. Depletion of such cells seems to deprive the patient of one of his most important natural defense mechanisms. Nevertheless, all mice in such experiments survived the depletion without aggravation of their health status. Even neutrophil depletion for up to one week did not lead to death of the animals (data not shown).

Obviously, such treatment is not possible for human patients. Consequently, depletion of neutrophils in patients should be tumor specific and a complete systemic depletion should be avoided. For instance, the infiltration of neutrophils could be prevented by inhibiting the influx of such cells. If TNF-α is the trigger for the induction of increased permeability of the tumor blood vessels and neutrophil infiltration, the prevention of its production or its neutralization can be a possible direction. However, if influx of blood is important for necrosis establishment and enhancement of therapy, neutralization of TNF-α might be deleterious for the intended treatment.

A specific alternative would be to engineer the tumor-targeting microorganisms to secrete neutrophil-depleting or -inactivating reagents. For instance, specific antibodies could be employed or toxins that bind to cellular receptors, which are only found on neutrophils.

Nevertheless, to optimize bacterial tumor therapies it is necessary to further investigate the exact mechanism how neutrophils interact with bacteria inside the tumor and/or with infected or necrotic cells, respectively.

Generally, the depletion of neutrophils from solid tumors and the prevention of formation of a neutrophil border appears to be a promising way to promote bacterial spreading and to ensure equal distribution of bacteria-carried, therapeutic agents inside the tumor. This should support efficiency and success of all kinds of bacteria-mediated tumor therapies.

## 4.3 Biofilm formation and encapsulation inside solid tumors

An unexpected outcome of the present analysis was the detection of encapsulation and biofilm formation by *S. typhimurium* SL7207 and *S. flexneri* Δdap inside solid CT26 tumors. Such a phenomenon had not been reported before and might severely influence the efficiency of bacteria-mediated tumor therapies.

The sheet-like structures that could be observed for *S. typhimurium* SL7207 strongly resemble the multicellular appearance that was described by Romling, et al. (Romling, et al., 1998)) for *S. typhimurium in vitro*. Interestingly, *S. flexneri* Δdap also display multicellular behavior inside solid CT26 tumors, although both, biofilm and capsules are different from those of *S. typhimurium*. *S. flexneri* was encapsulated in long, tube-like structures, which were apparently open on both ends and most likely enclosed several bacteria. In contrast, most of the *Salmonellae* seemed to be individually encapsulated and the majority of capsules detected for *S. typhimurium* were closed on both sites.

In contrast to both of the above described bacterial strains, the laboratory strain *E. coli* TOP10 did not show any capsule or biofilm formation. TOP10 is a K12-derivative and Romling et al. (Romling et al., 2005) reported that several K12-derivatives had lost their ability to produce a biofilm. Thus, the present data correlate well with such data.

The observation that biofilm formation and encapsulation is different for *S. typhimurium* SL7207 and *S. flexneri* Δdap is giving some clues on the induction of such structures. While *Salmonellae* showed an impaired multicellular behavior in neutrophil-depleted CT26 tumors, the behavior of *Shigellae* was not affected by the presence or absence of such cells. This suggests that for *Salmonellae*, multicellular behavior inside solid tumors is a reaction towards the host's immune system. It might represent an evasion mechanism that prevents phagocytosis by host neutrophils. In addition, bacterial cells in communities are better protected against deleterious agents (Costerton, 1997), which might be in case of tumor colonizing *Salmonellae* antimicrobial peptides that are produced by neutrophils. However, this preferred multicellular growth inside solid tumors probably keeps them from infecting viable tumor cells and thus might represent an obstacle for DNA transfer or the efficient delivery of therapeutic proteins.

On the other hand, the property to form capsules and biofilms might take place exclusively in tumors and not in other tissues. This implies that tumor specific bacterial promoters exist, which should allow a highly specific tumor-restricted expression of therapeutic or modulating genes like genes encoding toxins or neutrophil-depleting agents.

The multicellular behavior of *S. flexneri* Δdap is apparently not dependent on neutrophils. Thus, both biofilm formation and encapsulation by *Shigella flexneri* might primarily be induced by the tumor microenvironment as biofilms generally can improve the metabolic state by trapping the nutrients (Costerton et al. 1999). The tube-like structures with open ends on both sites might provide the possibility for the bacteria to change their status from sessile to migratory by leaving the protective tubes and colonize areas outside necrosis. This would be in agreement with the findings that single *Shigellae* were found in vital tumor tissue.

Nevertheless, a general protective role of such structures against the immune system of the host should not be excluded.

The biochemical composition of the observed capsules and biofilms is still unresolved. *In vitro* studies with biofilm-producing *S. typhimurium* and *E .coli* (Zogaj et al., 2001) revealed that two major components of biofilms produced by *S. typhimurium* could be thin aggregative or curli fimbriae and cellulose. Co-production of both substances lead to the formation of a highly hydrophobic network with tightly packed bacterial cells that accumulated in a rigid matrix. Electron micrographs of such biofilms strongly resembled the biofilms observed for *S. typhimurium* in CT26 tumors. Thus, cellulose and curli fimbriae are most likely also the components of the *Salmonella*-derived biofilm described here.

Other unresolved questions are, for instance, the influence of the multicellular behavior on bacterial dissemination and on bacteria-host interactions. In general, it is unclear whether this behavior is beneficial or detrimental for bacterial tumor therapies.

All these questions need to be addressed. Thus, genomic and biochemical approaches should be employed to characterize the nature of the capsule and the biofilms. This is a prerequisite for establishment of appropriate deletion mutants that allow the answering of such questions. It will also unequivocally show whether biofilms are the major cause for the low efficiency of bacteria-mediated gene transfer into tumor cells *in vivo*.

## 4.4 Bacterial gene transfer into solid tumors

Transfer of eukaryotic genes into mammalian cells by different Gram-negative and Gram-positive bacteria has been reported repeatedly (Sizemore et al., 1995; Courvalin et al., 1995; Darji et al., 1997). Even a transgene expression in murine melanomas after infection with *Salmonella choleraesuis* has been claimed (Lee et al., 2004; Lee et al., 2005). Despite these promising reports, bacterial gene transfer is associated with a number of restrictions such as low efficiency and the difficulty to distinguish eukaryotic from prokaryotic gene expression.

By cloning an intact intron into the eukaryotic reporter gene, the difficulty of distinguishing mammalian from bacterial gene expression could be successfully overcome. This system allowed the unequivocal proof of effective bacterial gene transfer by *S. flexneri* Δdap into different tumor cell lines *in vitro*. Bacterial gene transfer by these bacteria was highly reproducible *in vitro*. *In vivo* experiments with CT26-bearing BALB/c mice initially indicated functional transgene expression inside solid CT26 tumors. However, later experiments were met with fluctuating results. Investigations of bacteria-host interactions in the tumor by

histology and electron microscopy provided some reasons that might be responsible for such results.

Immune histology suggested that gene transfer is taking place *in vivo*, as a few cells in each tumor section were strongly positive for the reporter protein. Whenever a tumor cell in such sections was positive, it was surrounded by *S. flexneri*. However, the number of luciferase expressing tumor cells per tumor was extremely low. Hence, the total amount of luciferase was probably too low to be detected by any other method. By extrapolation, when transferring genes encoding therapeutic molecules into tumor cells using *Shigellae* under the present conditions *in vivo*, expression would most likely have only little effect, if any, due to the limited number of transfected tumor cells.

The main reason for low gene transfer efficiency by *S. flexneri* Δdap into solid CT26 tumor *in vivo* could be the poor dissemination of the bacteria inside the tumor. The majority of bacteria were found to accumulate inside large necrotic areas associated with dead cells and only a few *Shigellae* were found neighboring vital cells, into which gene transfer is possible. The depletion of neutrophilic granulocytes did not enhance gene transfer efficiency although more bacteria were found inside vital tumor tissue. However, the total number of vital tumor cells was drastically reduced under these conditions. Therefore, the number of transfected tumor cells might still be too low to become detectable.

A second reason for the fluctuating efficiency of *S. flexneri* Δdap-mediated gene transfer *in vivo* could be the encapsulation and biofilm formation by *Shigellae.* All *Shigellae* detected here are encapsulated and are not in direct contact with vital tumor cells. Thus, gene transfer cannot take place. However, the evidence of gene transfer into single tumor cells in the vital tumor tissue suggests that the few *Shigellae* inside vital tumor tissue are not encapsulated or enclosed in a biofilm and therefore are able to infect tumor cells and transfer genes.

In summary, it becomes clear that bacteria-mediated gene transfer might not be the optimal bacterial therapy for solid tumors under the present circumstances. While it still might be the method of choice for genetic vaccinations or for gene therapies into organs different from tumors, it is currently not yet appropriate for transfer of genes into tumor cells *in vivo*. Nevertheless, it should not be completely disregarded as a future way of tumor therapies, although priorities for further development of bacterial tumor therapies should lie on bacteria-produced proteins as promising alternatives.

## 4.5 Outlook

Consequently, future directions of research should aim at the construction of genetically engineered bacteria that are able to stably produce sufficient amounts of therapeutic molecules. Some of such molecules like CD are already active when residing in the bacterial cell. However, most therapeutic molecules are likely to be proteins that exert their activity by acting directly on the tumor cells. Thus, efficient secretion systems need to be established. This may be an ambitious task since bacterial secretion systems exhibit high specificity for the molecule secreted and the bacterial strain employed. Therefore, several of the possible systems have to be tested to meet the required demands.

An obvious application of an efficient secretion system would be the delivery of anti-neutrophilic compounds such as neutrophil-specific toxins or antibodies. Both strategies should help to kill or inactivate neutrophils locally without impairing the general health status of the patient. This should facilitate bacterial spreading in solid tumors and enhance necrosis and death of tumor cells. An enhanced bacterial dissemination in the tumor should also allow the bacteria to carry additional therapeutic proteins into the tumor.

Another option that needs to be tested urgently is the use of bacteria that are deficient in LPS. These could be LPS-deficient mutants or Gram-positive bacteria. At present it is unclear, which factors cause the release of neutrophil attractants and lead to the infiltration of neutrophils. LPS might be such a candidate. Thus, deficiency in LPS might prevent neutrophil attraction without limiting the formation of necrosis.

To ensure a tumor specific expression of such therapeutic molecules, but also of neutrophil-specific toxins or antibodies, the inquiry of tumor specific promoters is an obvious choice. The promoters should be activated exclusively inside solid tumors and thus prevent unwanted expression in tissues different from the tumor. The probability of the existence of such promoters is rather high. For instance, the formation of bacterial capsules and biofilms inside mammalian hosts has up to now only been found in solid tumors and has to be triggered by promoters that specifically respond to the conditions in the tumor. Alternatively, promoters, which are active only under anaerobic conditions, might be employed.

At present, the consequences of bacterial biofilm formation in solid tumors are unclear. As encapsulation and biofilm formation might influence bacterial dissemination inside tumors and, very likely, impair their ability to invade tumor cells *in vivo*, the investigation of bacterial mutants that are unable to form capsules and biofilms could help to improve our knowledge about bacteria-tumor interactions. Another option of similar kind is the use of different *E. coli* strains, which do or do not have the ability to build biofilms (Zogaj et al., 2001). Additionally, non-biofilm-forming *E. coli* might be engineered to produce invasin of *Yersinia enterolytica*.

The production of invasin renders them invasive and might influence their behavior in solid tumors.

The employment of gene transfer via bacteria into tumor cells should not be disregarded. The possibilities listed so far aim at the improvement of bacterial dissemination in the tumor and at an improved contact of the bacterial carrier with live tumor cells. Both strategies might already enhance DNA transfer. An additional modification that might result in improved bacterial gene transfer is the introduction of a gene encoding listeriolysin O of *Listeria monocytogenes.* This should help intracellular bacteria like *Salmonella* or invasive *E. coli* to escape into the cytosol and allow them to deliver plasmid load.

Besides different bacterial mutants, additional bacterial species could be investigated. One potential candidate is *Agrobacterium tumefaciens*. This bacterium is known to transfer genes into cells of plants and has been shown to transfer DNA into HeLa cells *in vitro* (Kunik et al., 2001). Interestingly, this bacterium is able to deliver DNA without invading the host cell. Recently, *Agrobacterium tumefaciens* has been shown to be able to target and proliferate in solid tumors in mice (Loessner, unpublished results).

In summary, further investigations based on the findings of this work, will very likely help to improve bacterial tumor therapies and might even render them a powerful alternative or additive for conventional tumor therapies.

# 5 Summary

Systemic administration of facultative anaerobic bacteria into mice that bear a solid tumor leads to a preferential accumulation of the microorganism in the tumor. Although facultative anaerobic bacteria have already been tested in experimental anti-cancer therapies for several decades, only little is known about the mechanisms that are involved in bacterial colonization of solid tumors and about bacteria-host interactions inside the tumor.

The present work deals with several aspects of bacterial tumor colonization and the impacts of bacterial settlement (I) on infiltration and composition of cells of the immune system, (II) on interactions of bacteria with such cells and (III) on influences of these interactions and of the tumor microenvironment on the bacteria and their behavior.

The originally tested bacteria-mediated gene transfer as a means of bacterial tumor therapy was strongly impaired inside solid tumors due to a strong increase of the size of necrosis after bacterial infection. Subsequent investigations lead to a better understanding of early bacterial settlement inside solid tumors. Short time after systemic administration of bacteria, single bacteria find their way into the solid tumor, where they start to form micro-colonies. With time such micro-colonies become larger and the bacteria start to spread. Concomitant is a strong influx of blood and neutrophilic granulocytes into the tumor. The mechanism that causes this influx is yet unknown, but experiments suggest a bacteria-induced release of neutrophil chemoattractants by tumor cells or immune cells here. This can result in increased blood vessel permeability and in the attraction of neutrophils.

The region of the tumor that had contact with the inflowing blood eventually becomes necrotic and was found to be surrounded by a barrier of host neutrophils. From this time on, bacteria are only found inside the necrotic areas, enclosed by a border of neutrophilic granulocytes.

By depleting host neutrophils, dissemination of bacteria inside the tumors and into vital tumor tissue could be enhanced. Besides a noticeable higher total number of bacteria inside the tumor an increase of the size of necrosis could be observed. Thus, manipulating tumor infiltrating neutrophils is one option to improve bacteria-mediated tumor therapy.

Further experiments unveiled an unexpected encapsulation and the formation of extracellular structures by *S. typhimurium* SL7207 and *S. flexneri* M90T inside CT26 tumors, but not by *E. coli* TOP10. While encapsulation and biofilm formation observed for *S. typhimurium* SL7207 was found to be dependent on the presence of neutrophilic granulocytes, no such correlation

could be observed for *S. flexneri* M90T. This suggests at least two different mechanisms that drive tumor-colonizing bacteria into the escape route of a protective biofilm. Mechanisms of development, composition and effects of bacterial biofilms inside solid tumors are yet unknown. However, their future revelation and the further extension of the findings of this work will help to improve bacterial tumor therapies and could give rise to a robust clinically relevant use of bacteria in anti-cancer therapies.

# 6 Abbreviations

| | |
|---|---|
| 5-FC | 5-fluorocytosine |
| 5-FU | 5-fluorouracil |
| 5'-UTR | 5'-untranslated region |
| BAK | BCG-activated cells |
| BCG | bacille calmette guerin |
| bp | base pairs |
| CD | cytosine deaminase |
| CFU | colony forming units |
| CIS | carcinoma in situ |
| CMV | cytomegalovirus |
| *c-onc* | cellular oncogene |
| CT | *Cholera* toxin |
| DAEC | diffusely adherent *E. coli* |
| DAP | diaminopimelic acid |
| DC | dendritic cells |
| DNA | deoxyribonucleic acid |
| EAEC | enteroaggregative *E. coli* |
| EGFR | epidermal growth factor receptor |
| EHEC | enterohemorrhagic *E. coli* |
| EIEC | enteroinvasive *E. coli* |
| ENA | epithelial-derived neutrophil attractant |
| EPEC | enteropathogenic *E. coli* |
| ETEC | enterotoxigenic *E. coli* |
| FCS | fetal calf serum |
| gro-α | growth-related oncogene α |
| Hly | hemolysin |
| HPV | human papilloma virus |
| IFN | Interferon |
| IL | interleukin |
| IM | inner membrane |
| IP-10 | interferon γ inducible protein 10 |
| i.p. | intraperitoneally |
| i.t. | intratumorally |
| i.v. | intravenously |
| LAK | lymphokine-activated killer cells |

| | |
|---|---|
| LD | lethal dose |
| LLO | listeriolysin O |
| LPS | lipopolyteichoic acid |
| LT | heat-labile toxin |
| mAbs | monoclonal antibodies |
| MCP-1 | monocyte chemoattractant chemokine 1 |
| MIC | monokine induced by interferon γ |
| MIP-2 | macrophage inflammatory protein 1 |
| MHC | major histocompatibility complex |
| MNEC | meningitis/sepsis-associated *E. coli* |
| MOI | multiplicity of infection |
| mRNA | messenger RNA |
| NE | neutrophil elastase |
| NK | natural killer cells |
| OM | outer membrane |
| OVA | ovalbumin |
| PAMP | pathogen associated molecular pattern |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PDGF-R | platelet-derived growth factor |
| p.i. | post infection |
| RCC | renal cell carcinoma |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| RT | room temperature |
| SCID | severe combined immune deficiency |
| shRNA | small hairpin RNA |
| SPI | *Salmonella* pathogenicity island |
| ST | heat-stabile toxin |
| SS | secretion system |
| SV40 | simian virus 40 |
| TA | tumor antigen |
| TE | trypsin-EDTA |
| TLR | toll-like receptor |
| TNF-α | tumor necrosis factor α |
| TSB | tryptic soy broth |
| UPEC | uropathogenic *E. coli* |
| VEGF | vascular endothelial growth factor |
| *v-onc* | viral oncogene |

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# Danksagung

Die vorliegende Arbeit wurde am Helmholtz Zentrum für Infektionsforschung (HZI) Braunschweig, in der Arbeitsgruppe Molekulare Immunologie angefertigt und wurde von der deutschen Krebshilfe e.V. finanziert.

Ich danke dem Mentor dieser Arbeit, Herrn Prof. Dr. Jürgen Wehland, für die Betreuung des Promotionsverfahrens. Ebenso danke ich Herrn Prof. Dr. Stefan Dübel für die Übernahme des Korreferats. Mein Dank gilt auch Herrn Prof. Dr. Norbert Käufer für seine Bereitschaft, als Prüfer zur Verfügung zu stehen.

Ganz besonders bedanke ich mich bei Herrn Dr. Siegfried Weiß, in dessen Arbeitsgruppe diese Arbeit angefertigt wurde, für die interessante Themenstellung, die großartige Betreuung während der gesamten Arbeit, seine stetige Diskussionsbereitschaft, seine Anregungen und Ideen, die wesentlich zum Gelingen dieser Arbeit beitrugen. Nicht unerwähnt lassen möchte ich hier sein Engagement, allen Mitarbeitern durch die Einrichtung von Seminaren und ‚journal clubs', sowie einem ‚brainstorming' im Harz eine ausgezeichnete Ausbildung und einen steten Überblick zu den aktuellen Fragestellungen im Bereich der Immunologie zuteil werden zu lassen.

Herrn Dr. Manfred Rohde danke ich für die Erstellung der elektronenmikroskopischen Aufnahmen.

Bei Frau Anna Link möchte ich mich für Erstellung und Färbung der Paraffinschnitte bedanken, Herrn Dr. Reinhard von Wasielewski danke ich für seine Hilfe bei der Analyse und Auswertung der Paraffinschnitte.

Allen ehemaligen und derzeitigen Mitarbeitern der Arbeitsgruppe Molekulare Immunologie möchte ich für ihre ständige Hilfsbereitschaft und das äußerst angenehme Arbeitsklima danken. Mein besonderer Dank gilt dabei Frau Susanne zur Lage und Frau Regina Lesch für die hervorragende technische Unterstützung im Labor.

Danken möchte ich insbesondere auch Herrn Dr. Holger Loessner und Frau Sara Leschner für ihre Unterstützung, Diskussionen und Anregungen während dieser Arbeit. Bei Frau Dr. Andrea Zelmer und Frau Dr. Jaga Jablonska bedanke ich mich für die Einweisung in sämtliche Arbeitstechniken.

Dem gesamten Team des Tierhauses danke ich für die gute Zusammenarbeit und die Pflege meiner Mäuse.

Besonders bedanken möchte ich mich auch bei meinen Eltern für die stetige Motivation und Unterstützung während meiner Zeit als Doktorandin, insbesondere meinem Vater für das große Interesse an meiner Arbeit.

Mein ganz besonderer Dank gilt Marc, der mir bei allen Problemen zur Seite stand, mich unterstützt und aufgemuntert hat und der immer für mich da war.